

**Protocols**  
*for an*  
**All Taxa Biodiversity Inventory**  
*of*  
**Fungi**  
*in a*  
**Costa Rican Conservation Area**

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## **Foreword:**

# **Inventory—Preparing Biodiversity for Non-damaging Use by Society**

Costa Rica has been a major arena for tropical wildland biodiversity studies for centuries. In the 1980's this work began to focus on an emerging philosophy of "Save It, Know It, Use It" (e.g., Janzen and Gámez, 1997) as the only pragmatically viable hope for conserving large blocks of tropical wildland biodiversity into perpetuity. In late 1992, at an international meeting hosted by the Organization for Tropical Studies at the La Selva Biological Station in Costa Rica, Jim Edwards of the National Science Foundation (NSF) expressed what many had been thinking. Is it possible to do a serious inventory of a large complex piece of biodiversity, and is the scientific community ready to take on such a challenge?

In the spring of 1993, NSF supported a 3-day workshop to address this question generically. Fifty-five scientist/administrators concluded that, yes, it was, for a budget in the \$100 million range, in a minimum of seven years, and with enormous—shall we say unprecedented—collaboration among the taxasphere (Janzen, 1993), biodiversity administrators, and the user community (Janzen and Hallwachs, 1994). Such a budget would clearly cover at best 20% of the total real costs, underlining that such an All Taxa Biodiversity Inventory (ATBI) would have to be a highly collaborative synergism among many agendas and many players. A particularly heavy contribution of sweat equity and institutional resources would be necessary from the taxasphere.

In the same year, NSF funded the initiation of a directorate for a first ATBI of the Area de Conservación Guanacaste (ACG) in northwestern Costa Rica by INBio, the ACG and the taxasphere. NORAD (Norwegian Agency for Development Cooperation) then rose to the challenge of supporting a year of taxon-specific on-site planning workshops to address a very specific question:

Dear TWIG (Taxonomic Working Group):

If you are confronted with the immense challenge of a total inventory of the species-level biodiversity of "your taxon" in the 120,000 ha ACG (a complete dry forest ecosystem and adjacent rain forest and cloud forest), what would you do - in what time framework, with what budget, with what human resources, and to produce what products?

This question was addressed by the Hymenoptera, Coleoptera, Vertebrate, Nematode, Fungus and Mollusc TWIGs (Janzen, 1996a, b). This book is the \$31 million response of the Fungus TWIG to that challenge for their estimated 50,000 species of fungi in the ACG.

Subsequent combinations of economics and politics have since closed down the specific Costa Rican ATBI of the ACG by the ACG, INBio and the taxasphere. However, the planning process e.g., this book of protocols, is intended to be a contribution to the planning of any ATBI of any large complex block of wildland biodiversity.

We anticipate that ATBIs and ATBI-like activities will become progressively more frequent as nations prepare their wildland biodiversity, and its associated ecosystems, for non-damaging use by a very wide range of local, national, and international users. While the emphasis in this book is on getting the information to hand and eye, we suspect that a major follow-on process will be the presentation of this information through the Internet, effectively through biodiversity Yellow Pages at web sites. Certainly each species of fungus deserves at least one Species Home Page, if not more.

The global biodiversity management and development process is particularly indebted to Amy Rossman and the Fungus TWIG of the ATBI of the ACG for carrying their report through to presentation in this “final” form. This form is certain to continue to evolve through planning, and hopefully, through real world application as well. But what really matters is that an important portion of the scientific community dealing with a huge, complex and critically important group of organisms is rising to the challenge of massively preparing wildland biodiversity for non-damaging use throughout society.

Dan Janzen and Winnie Hallwachs  
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## Introduction and Overview

Fungi constitute the most diverse group of eukaryotic organisms on earth—second after the insects in the number of species thought to exist. They are united by a mode of nutrition in which they grow through the substrate secreting degradative enzymes and absorbing nutrients through their walls. Because they obtain their nutrients through absorption, they can successfully exploit all kinds of organic matter. They play a myriad of roles within the world's ecosystems among which the most important may be the cycling of nutrients derived from the breakdown of plant and animal matter allowing the re-use of scarce biotic and abiotic resources.

As heterotrophic organisms fungi may have a detrimental effect as, for example, in the cases of those that decay plant products and cause food spoilage or that are parasites of plants and humans. Alternatively, they may be beneficial as in the case of mycorrhizae—the fungus-root association essential for nutrient uptake in the majority of vascular plants. Fungi can be extremely useful such as in the biological control of noxious weeds, insects pests, nematodes, and plant pathogenic fungi. Although commonly associated with their hosts, the effects of some fungi are not known as, for example, the microfungi inhabiting interior tissues of living plants, or the Trichomycetes, a fungal group that exists attached to the intestinal wall of insects. The influence of fungi may appear rapidly as when a disease attacks a susceptible agricultural crop. Conversely, fungi may affect the environment slowly as in the case of lichens that break down rocks over thousands of years. Many groups of fungi exist about which little is known including the relationship to their host or substrate, and their role in ecosystems.

Fungi are economically valuable and have many uses that are not generally recognized. It is therefore in our own economic interests to conserve fungal diversity, but doing so requires inventories. In the United States alone, edible mushrooms generate an estimated \$700 million in revenues annually and form the basis for a major industry worldwide. Other fungi are essential for making basic foodstuffs such as molds in cheeses and yeasts in bread as well as the production of alcohol both for human consumption and fuel. Fungi serve as a source of commercially important enzymes, pharmaceuticals, and other natural products that translate into a multi-billion dollar industry. Fungi also are increasingly used to ferment solid organic wastes into usable products such as food, methane and fertilizers. In agriculture, on the other hand, fungi cause billions of dollars in damage to crops in the field as well as during transit and stor-

age prior to marketing. At present, knowledge of fungal diversity is of particular importance in order to prevent the inadvertent introduction of non-native species in the process of transporting crops, horticultural, forest, and other plant products within and between countries. Fungi including lichenized fungi may also be useful indicators of environmental degradation and climate change. An increased understanding of the diversity and natural history of fungi will contribute dramatically to knowledge of the earth's biota, and will greatly strengthen initiatives to protect and use sustainably our natural resources.

### 1.1 Diversity of Fungal Species

Because of their widespread distribution and association with all organic and many inorganic substrates, the actual number of fungal species in existence is difficult to assess. The most widely accepted estimate of the number of fungal species in the world is 1.5 million (Hawksworth, 1991) based primarily on a ratio of fungi to vascular plant species of 6:1 observed in temperate areas. This ratio may be higher or lower in tropical regions but, as yet, tropical fungi are not known sufficiently to even speculate about their species richness. Hawksworth (1991) estimates that less than 5% of the 1.5 million species of fungi are described and characterized.

A ratio of macromycete species to vascular plant species of 3.5:1 was proposed by Cifuentes et al. (1997) based on L. R. Hesler's data from a lifetime of collecting in the Great Smoky Mountains (Petersen, 1979). Cifuentes et al. suggested that this ratio is more relevant to subtropic regions of the Western Hemisphere than is a ratio based on the flora and mycota of the United Kingdom. Given an estimated 10,000 vascular plant species in the ACG and using the Hesler ratio, an estimate of the number of macromycete species is 35,000.

The portion of well-known fungal species is small for several reasons related to the nature of fungi themselves. Fungi are composed of a threadlike vegetative structure called mycelium that usually exists immersed in soil or plant parts. The fungus may become visible only when reproductive structures are produced. Estimates of numbers of fungal species are based on direct observations of comparable areas over time. Direct observations, as opposed to isolation from substrates or indirect observation, can be made for only a limited number of groups of fungi, namely those that produce conspicuous or relatively long-lived sporocarps. Direct observation occurs at two scales or levels—macroscopically (including use of a hand lens) in the field and microscopically under a dissecting or compound microscope in the laboratory. Often fungal reproductive bodies are small or otherwise inconspicuous and are produced only sporadically. Recent studies on the diversity of microfungi measured by indirect observation suggest a high level of fungal diversity that can only be observed indirectly—in laboratory culture rather than in the field. Bills and Pol-

ishook (1994) obtained 300-400 species from each of four replicate samples of 1 ml particle suspensions of leaf litter from a Costa Rican tropical rain forest. Among the species isolated, 40-60% were considered rare or were unidentifiable. In addition, many fungi will neither grow nor produce reproductive structures in culture, thus hampering our understanding of fungal species presence.

## **1.2 Why an Inventory of Fungi?**

At present, there is no single place in the world from which all of the living organisms are known. Such knowledge is the intended result of an all-taxa biodiversity inventory or ATBI, as proposed by Janzen and Hallwachs (1994). In an ATBI, all or almost all the organisms in a relatively large, biologically diverse, terrestrial ecosystem would be discovered and described as fully as possible during five years of intense study. The wealth of information resulting from such a study would be integrated, for example, to determine interrelationships between organisms or characterized for potential usefulness to humanity. In addition, the knowledge gained from an ATBI, specifically the discovery and characterization of biological resources would provide the basis for their sustainable use and thereby could help slow the loss of these resources. Producing immediate revenue and recognizing their value as long term biological resources might protect biological resources from destruction (Reid et al., 1993). Some studies have made a moderate attempt to demonstrate that biological diversity is more economically valuable as a sustainable, long term resource than as a short term resource that ceases to exist after the quick payoff (Carr et al., 1993).

Up to now, most biological inventories have involved only one or two major groups of organisms, primarily large animals and vascular plants, and never have such speciose groups as insects or fungi been included in terrestrial inventories in anything but a cursory way. A group of biologists representing all organismal disciplines concluded that an all or almost-all taxa biodiversity inventory could be achieved with reasonable success in a five-year period for an estimated cost of about \$50-100 million dollars (U.S.), most recently projecting \$94 million (Janzen and Hallwachs, 1994). To have a basis for such projections, an ATBI was defined as encompassing a study area of about 50,000-100,000 hectares relatively rich in species diversity and including one major ecosystem.

The fungi as a group of organisms lend themselves to sustainable use because many species can be isolated non-destructively and grown in culture to be perpetuated and used indefinitely. Other groups of fungi such as obligate plant parasites and mycorrhizal species are difficult to obtain and propagate in pure culture, but, with care, can be harvested from the wild in a manner that does not destroy the parent germplasm. Thus fungi were selected as one of the groups of organisms to be included in a pilot program for an ATBI to take place within the Area de Conservación Guanacaste in Costa Rica (Janzen, 1996a).

### 1.3 History of Mycology in Costa Rica

The first collections of fungi in Costa Rica were made in the Central Volcanic Range by Anders S. Oersted in 1848. His specimens are now housed in Denmark and Sweden. In 1875 Helmuth Polakowsky issued several fascicles of fungi on plants collected mostly in the Central Valley (Meseta Central) and environs. See also Polakowsky (1877a, b). The largest collection effort in the Nineteenth Century was by J. Hemsley and collaborators in relation to Biologia Centrali America. These specimens were studied by M.J. Berkeley and M.A. Curtis as well as M.C. Cooke and G.E. Masee, all of whom described many new species from Costa Rica. Henri Pittier organized *Primitiae Flora Costaricensis* through a collaborative agreement with the Royal Herbarium in Brussels involving mycologists M. and Mde. Rousseau whose specimens are now in Brussels (Bommer and Rousseau, 1896; Hennings, 1902, 1904). At about the same time N.T. Patouillard received collections made by Tonduz (1896; 1897a,b) and published an account of these Costa Rican fungi (Patouillard, 1912). Tonduz also sent specimens, mostly plant pathogens, to Carlos Spegazzini in Argentina (Spegazzini, 1919).

The most important collector of lichens and agarics between 1910 and 1940 was Carroll Dodge. His specimens and notes were recently acquired by the Farlow Herbarium, Harvard University. The Costa Rican naturalist Alberto Brenes collected fungi and sent them to Hans and Paul Sydow who published the fungal exsiccata as A. Brenes' *Costa Rican Fungi* and Sydow's *Fungi in Itinerari Costaricensi lecti* (Sydow, 1925, 1926, 1927, 1928; Sydow and Petrak, 1929, 1937). Many Costa Rican specimens were included in Sydow's *Monographia Uredinearum*.

In 1966 W. C. Denison and his associates collected and published on Costa Rican Pezizales including one new genus and species from Guanacaste named *Geodina guanacastensis* Denison (1963, 1967, 1969, 1972). Goos (1960) reported on 47 species isolated from soils in Costa Rica and Panama. José Alberto Sáenz R. (University of Costa Rica) started his studies of Myxomycetes and Gasteromycetes in the mid-sixties; and María Isabel Morales Z. studied the Gasteromycetes of Costa Rica. Luis Diego Gómez started general fungal collecting in 1966 and was later joined by Rolf Singer working on the mushrooms of Costa Rica (Mueller, 1995). These studies are carried on today by Roy E. Halling (The New York Botanical Garden), Gregory M. Mueller (Field Museum), Clark L. Ovrebo (University of Central Oklahoma, Edmond), and others. Trained in Paris studying *Pleurotus* with Roger Heim, Ana V. Lizano is currently working on Costa Rican fungi at the University of Costa Rica. In 1970, Dra. Julieta Carranza initiated her study of Costa Rican polypores and now also works at the University of Costa Rica. Several students of Carranza have been

trained in mycology, namely Mariela Bermudez (endomycorrhizae), Armando Ruíz (Aphyllphorales), Milagro Mata (Agaricales), and Loengrin Umana (Agaricales). Milagro Mata is presently curator of fungi for INBio.

No comprehensive account of the fungi of Costa Rica has been attempted, even for individual groups of fungi. Following a two-month long collecting trip in Central America, Stevens (1927) reviewed the reports of fungi up to that time and listed 123 species, primarily Ascomycetes and Fungi Imperfecti, from Costa Rica and Panama. Morris (1972) described many hyphomycetous species new to science, several of which were known only from this publication until the work of Bills and Polishook (1994), Bills et al. (1994), and Mercado-Sierra et al. (1996, 1997). Based on the literature, Covington (1980) listed about 1,000 species of fungi reported from Costa Rica. At present, mycologists are actively working on the pore fungi (Carranza-Morse, 1991, 1992, 1993; Núñez, 1996), smut fungi (Piepenbring, 1995), foliicolous lichens (Lücking, 1993), and Agaricales. A search of computerized specimens in the U.S. National Fungus Collections revealed about 2,000 specimens representing about 1,200 taxa collected over the past 100 years (Farr, pers. comm.).

The ACG Fungus ATBI offers Costa Rican and Latin American mycologists an opportunity to participate in an exciting and challenging project that will generate opportunities for professional interrelationships as well as exchange of expertise and collections. A number of well-trained mycologists reside in Costa Rica as well as throughout the rest of Latin America (Appendix B).

## **1.4 Fungus Taxonomic Working Group (TWIG) Workshop**

The Fungus TWIG workshop to plan a Fungus ATBI for the ACG in northwestern Costa Rica was held on 9 through 19 June, 1995. It was attended by 25 collaborators from 10 countries (see page iii). All participants were experienced systematic mycologists; and, together, they represented expertise in diverse groups of fungi. In addition, a virologist served as an “outgroup” member and promoted the planning of interactive research with those studying other groups of organisms. As a result of the collective expertise, these contributors were able to develop protocols for a complete fungus inventory of the ACG and to make a preliminary assessment of the resources required to carry out the project. Two non-technical accounts of the workshop have been published, one by mycologist Paul Cannon (1995) and the other by the outgroup member and virologist Marilyn Roossinck (1995).

The objective of the workshop was to develop a detailed, sampling plan for conducting an inventory of all the fungi in the ACG.

## **1.5 Description of the Area de Conservación Guanacaste (ACG)**

The ACG is a reserve covering about 120,000 hectares in the northwestern corner of Costa Rica close to the Nicaraguan border. The elevation ranges from sea level to 2,000 m peaks; and vegetation varies from coastal mangrove swamps to seasonally dry forests; wet forests; and permanently very wet, cloud forests. The Area is divided roughly into eight vegetation zones as follows:

1. Mangrove - sea level
2. Dry Forest - volcanic tuff (100-500 m)
3. Dry Forest - serpentine soil (400-700 m)
4. Dry-Wet Forest Intergrade, Atlantic slope (500-800 m)
5. Wet Forest - Pacific slope (800-1,200 m)
6. Dry-Wet Forest Intergrade - northern Atlantic (200-400 m)
7. Rain Forest - (400-600 m)
8. Cloud Forest - (1,200-1,500 m)

For fungi associated with wood and terrestrial substrates, sampling protocols vary between vegetation zones with increased sampling of zones with higher expected numbers of fungal species.

The estimated number of vascular plant species in the ACG is 8-10,000 belonging to about 500 genera and 100 families, including approximately 1,200 tree species. Ideally all parts of each plant species in all life stages would be sampled both directly and indirectly. Because this is an unrealistic approach, protocols specify selected numbers of representative plant species to be sampled for fungi. Knowledge and expertise about the vascular plants in particular are needed by those working on the fungi, not only to determine the host identity but also to assist in selecting woody substrates as subjects for long-term sampling. Interaction with the Plant TWIG is essential. In addition, fungi occur in association with all kinds of organisms particularly insects and nematodes, but also protozoans, algae, non-insect invertebrates, fishes, birds, and mammals. Interactions with all of the other TWIGs (Janzen, 1996a) will be important for the Fungus TWIG.

## **1.6 Expected Number of Species**

After considerable discussion, the number of fungi expected to occur in the ACG was estimated at 40-50,000 species. This number was based on the ratio of fungi to vascular plant species developed by Hawksworth (1991) and assumes an estimated 8-10,000 vascular plant species in the ACG. This number

is corroborated by Rossman (1994) who estimated a total of about 40,000 fungal species as the summation of estimated species numbers in each major fungal group in a hypothetical Fungus ATBI of an area of 50,000-60,000 hectares which is about one-half the size of the ACG. However, this may be an underestimate based on the Hesler ratio for macromycetes (1.1) reported by Cifuentes et al. (1997). The confidence limit for the figure of 50,000 fungal species is largely unknown because most of the data that form the basis for the vascular plant-fungus species ratio originated from temperate ecosystems. Estimates based on direct long-term observations of comparable areas can only be made for a few fungal groups such as those with conspicuous, persistent fruiting bodies. Such estimates do not include fungi that can only be detected by indirect observation and are extrapolations from the long-term results of haphazard sampling strategy.

With the diversity of habitats and substrates present in ACG, many new fungi with unique properties and life histories are expected to be recovered. Based on the diversity of fungi yielded by one leaf litter sample from Costa Rica (Bills and Polishook, 1994), it is not unreasonable to expect 40% of fungi recovered from the Fungus ATBI to be undescribed.

Fungal diversity within specific ecosystems has only recently been widely contemplated, much less studied. Species-effort curves have rarely been calculated for fungi, and the few recent examples do not approach a complete fungus inventory. They were conducted over a short-term on fruiting bodies (Lodge and Cantrell, 1995) or on a single host species for endophytes (Lodge et al., 1996a) or on only two leaf litter species for microfungi (Polishook, et al., 1997). Additionally, two of the studies have been concerned only with macromycetes. In a field where rates of discovery of new fungal species are often directly related to the number of specialists participating, it is not surprising that no inclusive inventory of a mycota has ever been conducted on the scale proposed for the Fungus ATBI. The intensive systematic sampling of all substrates present in the ACG will undoubtedly result in the inventory of a high number of fungal species many of which will be new to science.

## **1.7 Sampling Protocols**

There is no universally applicable technique to assess fungal diversity or even locate all of the species present. For fungi the techniques for estimating and delineating species diversity have not been developed, much less evaluated for effectiveness. Because of their ubiquitous association with many kinds of substrates, their seasonal appearance and the limited existence of identifiable sporocarps, their often small size, and the difficulty or inability of obtaining the organisms in pure culture, fungi cannot be sampled using a single class of techniques. A range of sampling protocols must be available in order to begin to

approach a complete inventory of fungi [see Carroll (1995) and Lodge et al. (1996b)]. Such a range is outlined in these protocols. This will result in a certain amount of overlap of effort. The protocols include approaches for minimizing this overlap as sampling protocols are tested and revised.

In developing a plan to assess fungal diversity, it is necessary to consider the variety of substrates and hosts from which the fungi derive their nutrition. These substrates must be sampled over time in all stages of development using an array of techniques. In order to develop sampling protocols and outline the approaches to be used, the substrates have been categorized as: 1) living plants; 2) wood; 3) other terrestrial plant substrates and soil including plant litter, water, and rocks; and 4) animals and animal products. A sampling plan for each substrate category has been developed. For all categories of substrates, fungi are sampled systematically both by direct observation of fungal sporocarps and by indirect observation—the isolation of fungal colonies in pure culture. In addition, a molecular approach to sampling fungi has been proposed in an appendix to the Plan in the form of a one-year preliminary research project that might provide a rapid assessment of fungal diversity in a small area and might some day serve to spot-check the efficiency of sampling using more traditional means.

In order to maximize coverage of diverse habitats, the ACG is divided into vegetation zones. These will be systematically sampled in various ways. Habitats will be sampled with different intensities relative to the diversity of host plants and expected and actual numbers of fungal species obtained in preliminary sampling. Within these plots all relatively large sporocarps will be collected periodically from soil, litter, and wood. Portions of the litter and smaller substrates such as twigs and mosses will be removed to the laboratory for inspection with a dissecting microscope to locate sporulating microfungi. Living plants of species representative of plant families and growth forms associated with the transects will be inspected for fungi on leaves and other living plant parts. Purposely felled trees of selected host species will be sampled for corticolous fungi, lichens, foliicolous fungi from the canopy, and wood-associated fungi appearing as the trees decay. Soil cores and litter associated with transect plots and aquatic habitats will be sampled. All substrates will be sampled by removing substrate from the field and isolating fungi in the laboratory. A variety of culturing procedures and media will be used to isolate fungi in the laboratory. Once isolated, identification may require that the fungus be grown under specific light and growth conditions to induce sporulation.

Fungi associated with animals and animal products will be enumerated through a series of protocols also based on direct observation and by isolation of fungi from sampled animals and animal products. Because of the tremendous

diversity of animals, from protoctistans to mammals, these protocols range from burying cadavers followed by periodic examination for macrofungi to baiting soils with nematodes followed by microscopic observation for their fungal parasites, and making observations and isolations from animal products such as hair, bone, dung, and nest material incubated in moist chambers. In addition some fungi associated with animals, such as the Laboulbeniales specific to particular insect groups, may be observed directly by members of the other TWIGs studying these groups of animals, and shared with members of the Fungus TWIG.

## **1.8 Training and Capacity Building**

The challenge of inventorying the vast array of fungi in the ACG is considerable. Most of the responsibility for carrying out the fungal ATBI will be placed on Costa Rican scientists, either with or without a formal academic background, particularly the community of parataxonomists and curators associated with the Fungus TWIG. Developing local expertise will have the lasting benefit of ensuring the continued sustainable use and management of fungi and fungal resources in Costa Rica and the rest of Latin America. This approach serves to reduce the funds required to engage international experts and ensures that the activities related to the Fungus ATBI will continue once the experts leave the country. Training in-country experts also provides the basis for extending the fungus inventory to other parts of the country. Some international expertise is required both for training in-country personnel and specimen identification. Funding must be budgeted for the salaries and expenses of international experts.

## **1.9 Potential Products**

Numerous economic, educational, and scientific products will result from the Fungus ATBI that will benefit many aspects of Costa Rica and the global community. The products are summarized briefly in this section.

### **1.9.1 Agricultural Products**

- a) Cultivation of new, edible mushrooms and other edible fungi
- b) sustainable usage of naturally occurring forest fungi
- c) organisms and products for food processing, such as better fermentation strains and vegetarian sources of food processing enzymes
- d) products for alternative livestock feeds through delignification
- e) knowledge of the role of gut fungi in digestive processes of ruminants

- f) improved plant nutrition through fungal associations, e.g., mycorrhizae
- g) increased seed germination through fungal associations, e.g., in orchid culture
- h) control of plant diseases through better knowledge of fungal vectoring in conjunction with the Virus ATBI
- i) organisms that will convert agricultural wastes such as coffee pulp to animal feed
- j) potential biocontrol agents for plant and animal diseases.

### **1.9.2 Industrial Products**

- a) Organisms useful for biological control, promotion of plant growth, and regeneration of disturbed areas
- a) bioremediation of hazardous wastes and polluted areas
- b) environmental indicators of pollution and habitat change
- c) sources of natural products, including cosmetics, dyes for fabric and paper, products for food processing, products for paper and wood processing, enzymes for biotechnology, and specialty chemicals
- d) bioassays for specific metabolites.

### **1.9.3 Medical Products**

- a) Fungi that yield novel antibiotics and other medicinal compounds
- b) increased knowledge of human fungal diseases and potential animal models
- c) methods for the rapid identification of microorganisms, including emerging disease agents.

### **1.9.4 Educational Products**

- a) Field guides for mushrooms, polypores, and lichens, that can be used in primary and secondary education
- b) computer or video programs documenting diversity and natural history of unique and interesting fungal groups
- c) basic educational texts illustrating the intricate ecological associations of life forms

- d) local and regional education programs through field trips and lectures
- e) internship programs for secondary and college level students from Costa Rica and abroad
- f) development of local expertise in fungal taxonomy
- g) cultural and educational exchange between local and foreign participants in the Fungus TWIG
- h) creation of exportable skill base for development of fungal inventories of other countries throughout the world.

### **1.9.5 Ecotourism Products**

- a) Field guides to mushrooms, polypores and lichens (as above)
- b) natural history stories in the form of books and videos
- c) exhibits, such as a leaf cutter ant nest with its associated fungus garden
- d) materials for museum displays
- e) “coffee table” books with watercolor paintings and color photographs of beautiful fungi
- f) guided field tours for those interested in mushrooms and other conspicuous fungi.

### **1.9.6 Social Products**

- a) Training for community leaders and government officials in the potential benefits and risks of biocontrol
- b) development of environmental sensitivity, such as the essential interactions of plants with an array of fungal symbionts
- c) applications to forensic science, such as the use of “corpse-finder” mushrooms.

### **1.9.7 Research Community Products**

- a) A realistic measure of species richness
- b) protocols for assessing fungal diversity useful in other geographic areas and other inventory projects
- c) a fundamental understanding of evolution, especially the co-evolution of symbiotic associations (e.g., fungus-virus associations, fungus-plant associations, and fungus-insect associations)

- d) opportunities for multidisciplinary research
- e) evaluation of techniques for collection, enumeration and identification of fungal taxa
- f) identification guides, query-based data bases, and diagnostic keys especially for less conspicuous taxa; increased knowledge of fungal taxonomy
- g) a comprehensive culture collection of Costa Rican fungi
- h) estimates of fungal species diversity and methods for making such estimates cost effectively on a broad, geographic scale
- i) foundation for future work in ethnomycology and the discovery of fungal medicinal products.

## **1.10 Resource Needs**

The estimated cost of conducting an ATBI for fungi ranges from \$10-30 million depending on the extent to which specimens are identified by international experts. Assuming an expected 50,000 taxa, each taxon would be represented by an average of 20 specimens. Assuming maximal use of expert identifiers, costs associated with obtaining the specimens and making preliminary identifications are about \$30 million. This includes a minimal estimate of expenses for the education and salaries of in-country human resources as well as the supplies and equipment necessary to collect, process, identify, and store the fungal specimens encountered in the Fungus ATBI. Identification of the specimens by specialists is required. Assuming an average price of \$20 per specimen for an expert identification or verification, the additional expense for one-million specimens would be \$20 million. This sum comprises nearly two thirds of the total estimated cost; therefore, it is recommended that emphasis be placed on the development of substantial in-country expertise through training opportunities and development of identification aids. Using this approach, the expense associated with the identification of the anticipated one-million fungal specimens could be considerably reduced. The overall costs for the Fungus TWIG is estimated to be between \$200-600 per taxon.

## 2

# Conventions and Document Road Map

## 2.1 Conventions Regarding Description of Protocols

### 2.1.1 Structure of Protocols as Requirements, Recommendations, and Options

The protocols of this Plan are described as a series of requirements, recommendations, and options. A requirement states that a certain method shall be used or that a certain action or series of actions shall be taken. Requirements contain the word “shall.” A recommendation states that a method should be used or that an action should be taken. It is understood that this method or action is not required for the successful completion of the defined protocol. Recommendations contain the word “should.” An option gives permission for an action that is not otherwise forbidden to be taken. Options contain the word “may.”

### 2.1.2 Labeling of Sections

In order to facilitate communication concerning a document that is likely to exist in both Spanish and English, each requirement or recommendation is labeled and can be referenced uniquely by that label. This labeling also facilitates discussion and revision of the Plan.

Each section of the Plan is numbered. For example, the present section is section 2.1.2.

### 2.1.3 References to Requirements, Recommendations, and Options

A requirement or recommendation is identified by the section number in which it appears followed by the lower case letter associated with the requirement or recommendation. For example, the goals of the Plan are provided in requirement 3.1a; and the requirement for obtaining spore prints of macrofungi is requirement 6.2.1b.

Cross references in the text are by means of section, requirement, or recommendation numbers in parentheses. For example, this is a cross reference to the section on goals of the Plan (chapter 3), and this is a cross reference both to the requirement stating those goals and to the requirement for obtaining spore prints of macrofungi (3.1a, 6.2.1b).

Requirements, recommendations, and options may sometimes have subdivisions; these are marked with lower case Roman numerals. For example, the

requirement for development of stop rules during the pilot program is to be found in section 3.2c(ii).

### Requirements

- a The requirements, recommendations, and options in each Requirements section shall be contained in a single alphabetically indexed list. References to any requirement, recommendation, or option shall be written in the form:

15.1.1c(ii)  
 \_\_\_\_\_  
 Index of requirement, \_\_\_\_\_  
 recommendation or option  
 \_\_\_\_\_  
 Subindex (if any)

## 2.2 Terminology

Throughout this Plan, the word “substrate” is used to mean any material upon or in which a fungus may grow or to which a fungus may be attached. The word “host” is intended to include all living substrates—especially those that can be identified with certainty as being the substrate for a given fungus.

## 2.3 Document Road Map

This Plan is composed of five major parts:

- a) introductory materials and description of Fungus ATBI goals and management (chapters 1 through 3)
- b) sampling protocols (chapters 4 through 11)
- c) needs for training and resource capacity development (chapter 12)
- d) bibliographic and computer resources required for the Fungus ATBI (chapter 13)
- e) potential products that could be derived from the knowledge developed as a result of the Fungus ATBI (chapter 14)
- f) financial resources required for the Fungus ATBI (chapter 15).

The introductory materials consist of nine sections:

- a) a discussion of the diversity of fungal species (1.1)
- b) a description of the purposes of a Fungus ATBI (1.2)
- c) a brief history of mycological work in Costa Rica (1.3)

- d) a brief description of the Fungus TWIG Workshop (1.4)
- e) a brief description of the ACG (1.5)
- f) a brief discussion of the number of species expected to be encountered by the Fungus ATBI of the ACG (1.6)
- g) an overview of sampling protocols (1.7)
- h) an overview of issues of training and capacity building for the Fungus ATBI (1.8)
- i) a brief list of potential products of a Fungus ATBI (1.9).

The main body of the Plan addresses sampling protocols. An overview of these is provided in chapter 4. The layout of study plots for sampling is treated in chapter 5. Protocols are organized by the substrates on which fungi occur. Protocols common to the sampling of fungi from two or more kinds of substrates are segregated and presented first:

- a) Common protocols related to collecting will be found in chapter 6.
- b) Common protocols related to isolation and culturing will be found in chapter 7.

The substrate specific protocols are organized by chapter as follows:

- a) protocols for sampling fungi from living plants and fungicolous fungi (chapter 8)
- b) protocols for sampling fungi from dead or recently cut wood and woody substrates (chapter 9)
- c) protocols for sampling fungi from terrestrial and soil substrates (chapter 10)
- d) protocols for sampling fungi from animals and animal products (chapter 11).

Preliminary protocols proposed for sampling fungi using a molecular approach are included as Appendix C. Other appendices include a list of references valuable to parataxonomists and curators required to identify specimens to genus (Appendix A) and a list of active Latin American and Spanish mycologists (Appendix B).



### 3

## Goals and Management of the Fungus ATBI of the Area de Conservación Guanacaste, Costa Rica

### 3.1 Goals of the ACG Fungus ATBI

#### Requirements

a The following information shall be obtained:

i the list of species present with their substrates and localities

Note: While identification using an accurate scientific name is desirable, determining species presence may require only that each species be distinguished from every other species present.

ii one or more methods for locating each species during the Fungus ATBI and in the future

iii a beginning account of the natural history of each species.

b All information about the ACG Fungus ATBI shall be computerized and be in the public domain.

### 3.2 Time Frame of the ACG Fungus ATBI

#### Requirements

a The time frame of the ACG Fungus ATBI shall be seven years.

b The first two years of the ACG Fungus ATBI shall be dedicated to a pilot program (6.1.1a).

Note: Activities of the pilot program are described in the protocol sections of this Plan (e.g., 5.1, 8.5.9u through 8.5.9x).

c During the pilot program of the Fungus ATBI,

i the collecting, isolating, culturing, and curating protocols (chapters 5 through 11) shall be evaluated and revised as necessary

ii stop rules (3.3.2a, 3.3.2b) shall be devised as necessitated by the data collected and implemented.

d The third through the seventh years of the ACG Fungus ATBI shall be dedicated to a full-scale ATBI (6.1.1a).

3.3 Management of the ACG Fungus ATBI

3.3.1 Personnel and Responsibilities

Requirements

- a The organizational chart for the ACG Fungus ATBI shall be that provided in Fig. 1.

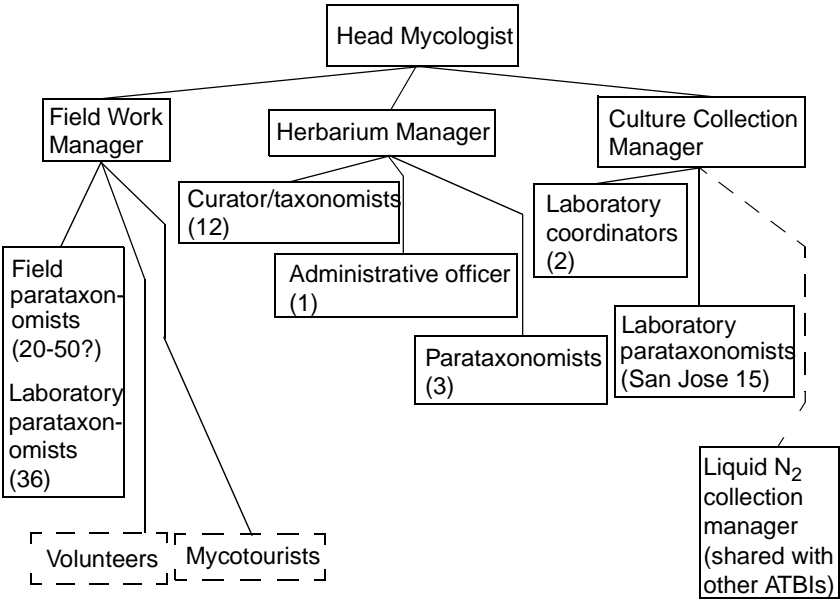


Fig. 1. Organizational chart for the ACG Fungus ATBI

- b The daily management of the ACG Fungus ATBI and the direction of work toward the goals of the ATBI shall be the responsibility of the Head Mycologist.
- c The direct staff of the Head Mycologist shall comprise the Field Work Manager, the Herbarium Manager, and the Culture Collection Manager.
- d The implementation of all collecting protocols shall be delegated to the Field Work Manager.
- e The implementation of all curation protocols exclusive of culture collection management shall be delegated to the Herbarium Manager.
- f The implementation of all culturing and culture maintenance protocols shall be delegated to the Culture Collection Manager.

- g All scientific work shall also be overseen by an Expert Taxonomists Group made up of members of the Fungus TWIG.
- h The Expert Taxonomists Group with the Head Mycologist and his/her direct staff shall have the responsibility for any alteration of collection, isolating, culturing, and curating protocols (e.g., the development, promulgation, and implementation of stop rules).
- i The Expert Taxonomists Group with the Head Mycologist and his/her direct staff shall have the responsibility for periodic review of the ACG Fungus ATBI to evaluate progress toward its goals, develop any remedial action necessary to improve performance, and implement remedial actions as may be defined.

### **3.3.2 Stop Rules**

#### **Requirements**

- a The function of stop rules shall be to define when the sampling for an organism or group of organisms may be converted to simply recording the presence/absence of such an organism or group or may be halted entirely.

Note: Stop rules that reduce collecting, but specify continued recording of organisms may be valuable. They should be defined for any sampling unit when commonly collected organisms become sufficiently familiar to field parataxonomists that the organisms can be identified at sight. Also, organisms for which vouchers are sufficient for a sampling unit and that can be identified in the field laboratory may not need to be preserved. An understanding of how to limit collecting or vouchering must be developed by taxonomic experts.

Note: Definition of detailed stop rules is beyond the scope of this Plan. Stop rules must be developed after results of sampling are analyzed.

- b A number of protocols require stop rules (3.3.2a) that cannot be developed prior to execution of an initial portion of the Fungus ATBI; these stop rules (8.4.1h, 8.5.4d, 10.1.1q, 10.1.4j, 10.1.4l) shall be developed in a timely manner once the process is initiated.

#### **Recommendations**

- c Stop rules (3.3.2a) should be defined for individual sampling units, multiple sampling units, habitats, or for the entire region of interest of the Fungus ATBI as appropriate on a case by case basis.

### **3.3.3 Management Practices**

#### **Requirements**

- a The Head Mycologist with his/her direct staff (3.3.1) shall have among their responsibilities the definition and management of

- i documented intermediate benchmarks that permit tracking of progress toward the goals of the Fungus ATBI
- ii risks facing the project and plans for dealing with them

Note: A preliminary set of risks is listed in 3.4.

- iii coordination with other TWIGs
- iv a mechanism for tracking development and cataloging of project documents
- v an effective mechanism for assessing and preventing sample loss due to failure to process the material within an appropriate time period in the laboratory
- vi an effective mechanism for assessing and preventing data loss including (1) mechanisms for computer and hardcopy backup of all field data and data bases and (2) checklists, forms, and other manual methods of data entry to prevent data loss should computers become unavailable temporarily for any reason
- vii balancing the work load of staff
- viii analyses of tools, methods, etc. at appropriate phases of the project
- ix with the support of taxonomic experts (12.1.1a(iv)) a plan for assessing project quality periodically (e.g., more than once per year) and associated methods of quality assurance.

### **3.3.4 Management Practices—Discussion**

It is beyond the scope of this Plan to detail every contingency that may occur during the Fungus ATBI and mandate an action in response; however, a few anticipated pitfalls are addressed.

When the full-scale ATBI begins, if all field collecting tasks are attempted simultaneously, the number of staff, volunteers, and mycotourists required might be as high as 150. Training this number of people to produce high quality collections will be difficult. For reasons of cost and quality, the judicious spacing of collecting tasks, the timely introduction of stop rules, and other means of

|  |   |   |    |    |    |
|--|---|---|----|----|----|
| Sampling units = 14  | Total sampling units (Chapter 5) = 52   |   |    |    |    |
| Avg. collectors/wk. rain = 33  | 70  | 75  | 75 | 60 | 35 |
| <b>2 year pilot program</b>  | <b>5 year full-scale Fungus ATBI</b>  |   |    |    |    |
| Macromycetes & Myxomycetes in plots & opportunistic (biweekly).          | First 14 monthly, slowed by stop rules.<br>2nd 12 triweekly.  | 2nd 12 monthly, slowed by stop rules.<br>3rd 13 monthly, slowed by stop rules.<br>4th 13 monthly. |    |    |    |
| Litter (monthly).<br>Soil (once per season).                             | while collecting in first quarter of sampling units is slowly reduced by stop rules, the remaining three-quarters of sampling units are started at the rates on left. |   |    |    |    |
|  | Aquatic (4 times per season).   |   |    |    |    |
| Actively fallen trees (biweekly).<br>Naturally fallen trees (bi-weekly). | First 14 monthly, slowed by stop rules.<br>2nd 19 triweekly.<br>3rd 19 monthly.   | 2nd 19 monthly, slowed by stop rules.<br>3rd 19 triweekly.  |    |    |    |
|  | Living plant parts other than from actively fallen trees (bimonthly). Assumes sampling 20% of plant taxa of region.   |   |    |    |    |

Fig. 2. Allocation of collecting tasks during the seven-year ACG Fungus ATBI and staffing of these tasks

control must be exercised. Even the possibility of extending the ATBI to eight, nine, or ten years in some form may be necessary. Also, it is undesirable to require a large number of parataxonomists for a short number of years only to have stop rules put many of them off the job just when their expertise has been developed.

The time line for collecting during the ACG Fungus ATBI (as diagrammed in Fig. 2) was developed to provide an example of one approach that stabilizes the number of parataxonomists over the length of the full-scale project. This approach approximately halves the field parataxonomist portion of the staff over what would be required if all tasks were to be started simultaneously at the end of the pilot program period. The sampling period is reduced in some intervals for the habitats and organisms (particularly terrestrial macrofungi) that are described by the Plan as being most labor intensive. Stop rules (3.3.2a, 3.3.2b) are projected to come into effect in one or two years and, by the third year of work in a given vegetation zone, effectively halve the number of collections required—permitting a collector to double the area being sampled in a day.

Sampling of macromycetes and Myxomycetes is not projected in this illustration to be carried out intensively on all 52 plots (chapter 6) simultaneously. The 52 plots are broken up into groups of 14, 12, 13, and 13; and work begins on these plots in a staggered manner. Similarly, sampling from actively fallen and naturally fallen trees is divided into sampling of three groups of sites, and the start of work in the three sets of sites is staggered.

Note that sampling from living plants other than actively fallen trees is not projected to occur during the pilot program.

### **3.4 Risk Assessment for the ACG Fungus ATBI**

Risks threatening successful completion of the ACG Fungus ATBI include

- a) lack of available required taxonomic experts
- b) inability to locate, train, or retain sufficient staffing
- c) inability to attract and train sufficient numbers of volunteers or mycotourists to supplement the field parataxonomists
- d) not being able to sufficiently train short term volunteers and mycotourists so that quality collections with appropriate annotation can be obtained from their efforts
- e) inability to achieve timely completion of tasks
- f) inability to achieve sufficient quality in preservation and curation of collections

- g) inability to achieve or maintain sufficient quality in annotation of collections from parataxonomists
- h) inability to meet the planned sampling schedule
- i) inability to develop appropriate stop rules (3.3.2a, 3.3.2b) rapidly enough to avoid wasting effort and material.



## Sampling Protocols—An Overview

No single technique or set of techniques can be used to locate all or even most of the fungal species in an area. Although two publications outline the habitats and techniques that could be explored (Hawksworth et al., 1996; Rossman, 1994), never before has a definitive sampling strategy been proposed for inventorying all fungi in an area. Such a strategy necessarily employs a wide range of techniques and unavoidably results in a certain level of overlap. Designing sampling protocols to obtain a majority of fungal species present in a defined geographic area is an innovative activity.

The overall strategy to carry out a Fungus ATBI in the ACG will involve taxonomic experts working with parataxonomists to collect samples in the field according to the protocols outlined here. Fungi are encountered either by direct observation of sporocarps and sporulating structures in the field or by indirect observation, i.e. isolation into culture from the diversity of substrates that exists. Well-trained parataxonomists will process the collected material and samples as well as isolate fungi using a variety of procedures. After collecting and processing fungal specimens, parataxonomists will sort specimens into major groups and make preliminary identifications of fungi that are relatively well known and for which identification tools exist. Field parataxonomists will be trained in both sampling techniques and the recognition of macrofungi and microfungi in the field. Laboratory parataxonomists will isolate fungi from substrates using axenic techniques, observe their growth, and make preliminary identifications. A herbarium and culture collection (7.5) at INBio in San Jose will be developed and managed by curators who may become international specialists in specific groups of fungi. Specimens and cultures of difficult and undescribed taxa will be sent to in-country and international taxonomic specialists. Experts in specialized taxonomic groups of fungi will be solicited for their cooperation in studying specimens from the ACG. Close cooperation between these experts and the curators and parataxonomists will be required for development of electronic data bases and identification tools. An extensive library of books and monographs will be essential for curators and parataxonomists to continue their training and develop in-country expertise (chapter 13, Appendix A).

Dimensions to consider when sampling for fungi are:

- a) Substrate - Organic matter in all stages of development and decay as well as some inorganic substrates support the presence of diverse fungi.

- b) Time - Most fungi produce their reproductive structures only irregularly and often ephemerally; thus, substrates must be sampled many times under varying conditions.
- c) Space - In tropical ecosystems as well as temperate rain forests, fungal-mediated nutrient cycling may occur entirely in the canopy; thus, above-ground substrates must be adequately sampled.

Substrates in all stages of development and decay must be sampled over time using an array of protocols. In order to enumerate the various approaches, the substrates have been categorized as: living plants, wood, terrestrial substrates including soil and water, and animals and animal products. A plan for each of the four substrate categories has been developed and summarized here. Details of the strategy for each substrate category are included in the following sections of this Plan. In designing the strategies, it was recognized that the distinctions between categories are not always clear; but it was decided that some overlap in sampling strategy is acceptable, even desirable. The four substrate categories are defined as follows:

- a) The section on fungi associated with living plants (chapter 8) includes fungi that exist as endophytes inside living plants, fungi that appear externally on living plant parts including foliicolous lichens, and fungi that occur on the bark of trees including corticolous lichens. Sampling of substrates in the forest canopy is considered in this section.
- b) The section on wood includes fungi associated with newly killed and decaying woody substrates (chapter 9) of all kinds including twigs over 1 cm diam and those fungi with perennial sporocarps.
- c) The section on terrestrial substrates (chapter 10) includes leaf litter and twigs less than or equal to 1 cm diam, soil, water, and rocks—particularly for lichens.
- d) The substrate category of animals and animal products (chapter 11) includes substrates such as protozoans, nematodes, insects, non-insect invertebrates, and fish as well as animal products and parts such as dung, fingernails, and hair, but also animal habitats such as nests and burrows. Some peculiar fungal groups are obligately associated with animals. In addition, these substrates are extremely diverse and the sampling strategies are generally quite different from those for the plant-associated substrates.
- e) Finally, a molecular approach to sampling fungi is suggested as a one-year preliminary research project to be conducted in an established molecular laboratory (Appendix C).

Sampling strategies for all substrate categories except animals and animal products are based on sampling units (chapter 5) within which plots to be sampled are placed at regular intervals (Fig. 3, Fig. 4). Within these plots all relatively large sporocarps are collected periodically from soil, plant litter, and wood. To locate sporulating microfungi, portions of the plant litter and smaller substrates such as twigs and mosses are periodically removed to the laboratory for inspection with the dissecting microscope. These substrates are also incubated in the laboratory to induce sporulation of a succession of microfungi. For identification and preservation as germplasm these fungi are isolated into culture from spores produced by these sporulating structures. To locate fungi associated with living leaves and other living plant parts, host species representative of plant families and growth forms associated with the transects will be selected and periodically inspected for sporulating fungi. In addition fungi on living plant specimens will be collected by those working on the Plant TWIG and contributed to the Fungus TWIG. To obtain fungi associated with both living and decaying wood over time, living trees representative of the vascular plant species present in ACG will be selected, purposely felled, and sampled for bark-inhabiting fungi including lichens, foliicolous fungi including fungi occurring in the canopy, fungi on associated epiphytic plants, and substrates that cannot easily be sampled from the ground. These trees will be monitored for years, possibly decades, to obtain the wood-associated fungi that appear as the trees decay.

All substrates including living and dead plant, fungal and animal substrates, soil cores, and aquatic habitats also will be sampled by indirect observation. Field samples are removed to the laboratory where fungi are isolated using a range of culturing procedures and media as outlined specifically in 7.2, 7.3, 8.5.1, 8.5.3, 8.5.5, 8.5.6, 8.5.8, 10.1.2, 10.2.4, 10.2.5, and 11.3.2. Appropriate procedures will be applied to diverse other substrates such as algae, mosses, lichens, and other fungi. The specific procedures used to isolate fungi can greatly influence the diversity of fungal species isolated, therefore, several different isolation techniques will be employed with emphasis on those that are known to favor the greatest diversity of fungal species. To obtain unusual species such as thermophilic and psychrophilic fungi, additional specialized techniques must be applied. Once isolated, identification may require that a fungus be grown under specific light regimes and growth conditions to induce sporulation. Isolates resulting from both the direct and indirect observations will rapidly result in vast quantities of collections and cultures that must be sorted, tentatively identified, and stored for future reference and use. INBio will maintain these products of the Fungus ATBI as voucher material and as a resource for future projects.

Fungi associated with animals and animal products will be located and isolated through a series of protocols also based on direct observation as well as

isolation of fungi from sampled animals and animal products. Because of the tremendous diversity of animals, from protoctistans to mammals, these protocols range from burying cadavers followed by periodic examination for macrofungi to baiting soils with nematodes followed by microscopic observation for their fungal parasites. Animals products such as hair, bone, dung, and nest material will be incubated in moist chambers from which a succession of fungi will be observed and isolated. Fungi specific to particular insect groups such as the Laboulbeniales and Trichomycetes may be observed on host specimens collected by members of the other TWIGs.

Members of other TWIGs should be alerted to the possible presence of fungi on their study subjects. This source of observation may be of particular importance in locating inconspicuous fungi on rarely examined substrates.

## 5

# Sampling Protocols—Establishment of Transects and Plots

The overall sampling strategy of the Fungus ATBI is based on a series of sites (each including one sampling unit) representative of the major vegetation types plus auxiliary plots selected to target specialized soil, parent materials, or stands of vegetation. For the two-year pilot program (3.2), 14 or 15 sampling units representative of the vegetation types will be established and sampled. For the five-year Fungus ATBI (3.2), 52 sampling units will be established with at least two sites within each vegetation type. The number of sites in each vegetation type was determined based on the anticipated diversity of fungi to be encountered in such vegetation. The entire ACG sampling is stratified by vegetation type (1.5); more area is sampled in particularly extensive habitats, e.g., dry forest, and those predicted to yield highest diversity, e.g., rainforest.

## 5.1 Transects and Plots for the Two-year Pilot Program

### Requirements

- a The basic terrestrial fungus sampling unit shall be referred to as a “sampling unit.”
- b A sampling unit shall consist of two parallel transects, fifty meters apart, 245 m long, with 4 m<sup>2</sup> circular plots (radius = 1.13 m) at 5 m intervals as illustrated in Fig. 3 and Fig. 4.

Note: Sampling of organisms with locally clumped distributions is most efficiently accomplished with dispersed sampling (Thompson, 1992). There is some evidence that this holds for terrestrial macrofungi (Lodge and Cantrell, 1995; O'Dell et al., 1995).

- c The starting point of each transect shall be marked permanently using a steel reinforcement bar enclosed within a segment of PVC pipe and marked with surveyors' plastic flagging.
- d The coordinates of each transect starting point shall be defined by GPS when a sampling unit is first established.

Note: Recording of the coordinates permits re-establishment of a sampling unit should all field markings for that unit be lost.

- e The permanent marker of each transect shall bear indelible annotation indicating the compass bearing of the transect.
- f The plots in a sampling unit shall be numbered as shown in Fig. 3.

- g For the two-year pilot program (3.2), 14 or 15 sites distributed according to Table 1 (representative of the vegetation types of the ACG (1.5)) shall be established.

## **5.2 Transects and Plots for the Five-year, Full Scale Fungus ATBI**

- a For the five-year Fungus ATBI, 52 sampling sites distributed according to Table 1 (representative of the vegetation types of the ACG) shall be established and permanently marked in the manner described in 5.1a through 5.1e.
- b The 52 sampling sites (5.1, Fig. 3) shall be identified and given a unique code in the gazetteer produced for the ATBI.

Table 1. Vegetation types and number of sites during seven year  
Fungus ATBI

| Habitat (ranked by area)   | Number of sampling units during Pilot Program   | Number of sampling units during 5-year Full Scale Project |
|--|---|---|
| dry forest (including temporarily disturbed areas in forest, such as those caused by landslides and creation of pasture)                         | 3<br>[ten 1m <sup>2</sup> squares in test of protocol for macromycetes, Myxomycetes, and lichens (10.1.1a through 10.1.1c)]       | 4@1, 2@ others  |
| dry lowlands (not serpentine)  | -   | 2   |
| dry serpentine (<400)  | -   | 2   |
| dry serpentine (400-700 m)   | 1   | 2   |
| dry-wet intergrade - Atlantic  | 1   | 4   |
| wet forest - Pacific   | 1   | 8   |
| dry-wet intergrade - Pacific   | 1   | 4   |
| rain forest - low  | 2 or 3*<br>[ten 1m <sup>2</sup> squares in test of protocol for macromycetes, Myxomycetes, and lichens (10.1.1a through 10.1.1c)] | 6   |
| rain forest - high   | 2   | 6   |
| cloud forest   | 2   | 4   |
| cloud forest - volcano   | -   | 2   |
| mangrove   | 1   | 2   |
| citrus   | -   | 2   |
| Totals   | 14 or 15 (10.1.1c)  | 52  |
| * The decision about the number of plots shall be made after a test of species overlap based on ten 1m <sup>2</sup> preliminary plots (10.1.1c). |   |   |

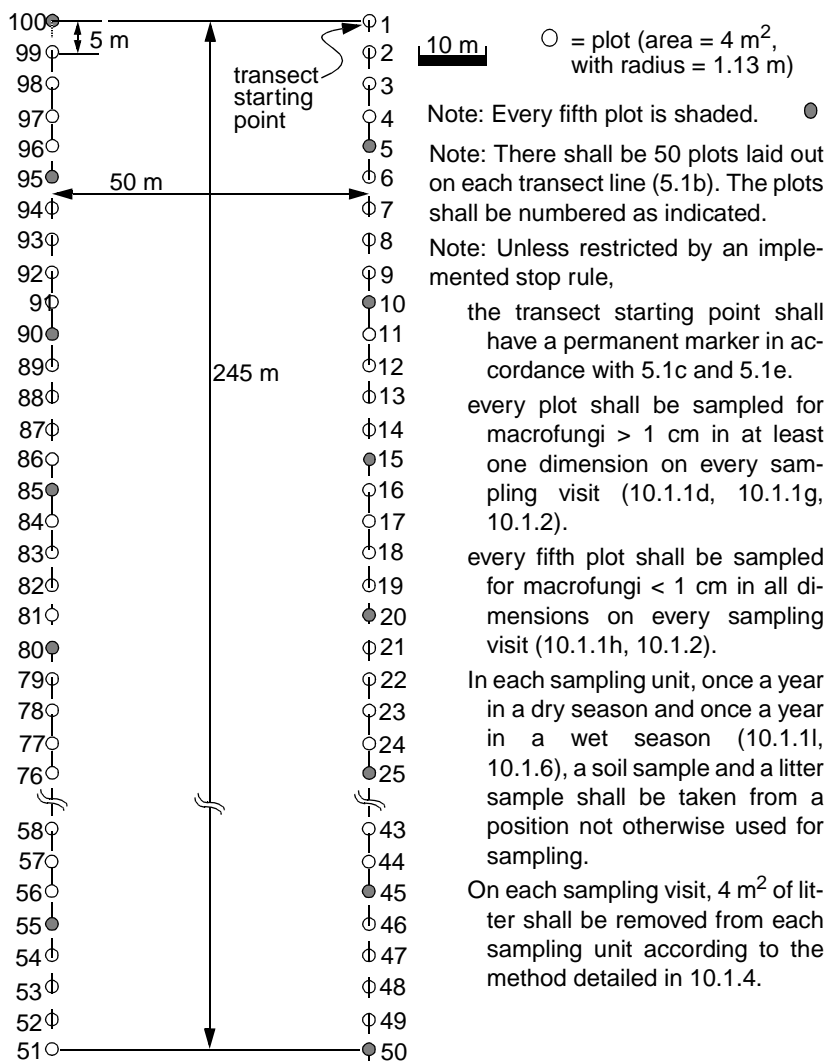


Fig. 3. Layout of transects for Fungus ATBI sampling of fungi

## 6

# Sampling Protocols—Common Procedures for Collecting and Handling Samples

## 6.1 Common Protocols for Collecting Fleshy Fungi, Fungi with Perennial Sporocarps, Lichenized Fungi, Microfungi, and Myxomycetes

### 6.1.1 Relevance of Protocols to Pilot and Full Scale Projects.

#### Requirements

- a All requirements, recommendations, and options of protocols in chapter 5, 6.1.2 through 6.4.1, and chapters 7 through 11 shall apply in both the pilot program and the full-scale ATBI described in this Plan unless otherwise explicitly stated within such a protocol.

### 6.1.2 Processing Collections of Fleshy Fungi, Fungi with Perennial Sporocarps, Lichenized Fungi, Microfungi, and Myxomycetes in the Field—Common Protocols

#### Requirements

- a When field processing of a collection is complete (6.2 through 6.4), the specimens shall be dried over low heat, i.e., 45 deg. C, for at least 12 hours and shall be stored in a sealed container so that the specimens do not rehydrate.

Note: With some fleshy taxa, it is important that they be dried rapidly in order to preserve anatomical characters of delicate structures such as lamellae. In such cases, a dryer with a built-in fan can prove invaluable. Smaller specimens can be placed in small wire boxes to prevent their being moved around by air from dryer fans. Some specimens, such as Phallales, must be dried within a few hours of collecting or they will simply dissolve.

Note: Further information concerning collection of lichenized fungi will be found in 8.4.3f through 8.4.3l, 8.5.3, 9.1.1c, 10.1.1, 10.1.5.

- b When spore prints are obtained, they shall be protected from heat, light, and mechanical damage and brought from the field sufficiently quickly for the spores to be viable on arrival at the laboratory.

## 6.2 Protocols for Collecting Macrofungi

Special procedures must be followed when collecting fleshy fungi in order to obtain sufficient deposits of viable spores and useful collections with rehydratable tissue and to record characteristics of the specimen essential for accu-

rate identification as well as for potential use in field guides and other references.

## 6.2.1 Collecting Macrofungi—Common Protocols

### Requirements

- a Macrofungi shall be carefully collected to keep sporocarps intact (e.g., the entire base or basal attachment structure shall be removed intact) and wrapped in foil or waxpaper or placed in a glassine or kraft paper bag.
- b From all macrofungi producing ballistospores, spore prints shall be collected on clean glass slides.

Note: Cultural studies derived from macromycete spores are beyond the scope of this Plan. Spore viability is variable and dependent upon species. Ascomycete spores could be grown in culture to identify anamorphs (7.3.1f(iv), 9.1.4e(ii)).

- c For species that produce ballistospores, appropriate portions of sporocarps shall be set up for spore prints (6.2.1b) in their wrappers (6.2.1a) in the field immediately upon collecting.

Note: This is an important step because a change in altitude between a field site and the laboratory can interrupt sporulation in some agarics (Halling, 1996). The present requirement maximizes the likelihood of obtaining a spore print.

- d During transportation from the field to the laboratory, sporocarps shall be protected from heat, light, and mechanical damage and fully processed prior to initiation of decay.

Note: It is extremely important that specimens be handled properly so that anatomical details required for determination are not permanently lost.

- e In the field, the following shall be recorded: field identification, sporocarp dimensions, color of all parts including basal mycelium and including any bruising or staining reactions (allowing up to 30 min. for color changes of context) or colors of tissues that change after sporulation (e.g., colors of lamellae in some agarics), texture, taste, odor, and relationship to substrate (such as position on a tree or fallen piece of wood).

Note: Position on a tree can be described using terms provided in 9.1.1d.

- f For smaller specimens, notes shall be taken on the following characters in the field: stipe features such as hairs, gill attachment, annulus and other veil features, and any other features that might be lost in transport.

- g In the field, characteristics of the substrate shall be noted such as size of branch, host identity, decay state of wood (9.1.1a), type of soil, or moisture content of soil.
- h Additional field notes shall be taken from both whole and sectioned specimens as required for tentative identification to genus (6.2.1e).

Note: Specification of genus-specific field note requirements is beyond the scope of this Plan. A partial set of important references can be found in Appendix A. Training of parataxonomists on genus-specific note taking requirements is key to obtaining specimens of macrofungi of greatest value.

- i Line drawings of sections that would help convey form and relation of elements of a fruiting body shall be made in the laboratory upon return from the field and shall be part of the specimen annotation.

Note: Examples of desirable illustrations are volval limb attachment, position of limbus internus of a volva, shape of lamellae, position and extent of region of context changing color on exposure to air or after application of reagent, annual or seasonal pore layers, form/position of stipe-columella, form/position of gleba, form of exoperidium, presence/position of endoperidium, presence/form of receptaculum, form/presence of indusium, form/presence of epiphragm, form/presence of peridioles, etc.

- j After return from the field, photographs shall be taken of the fresh specimens on a plain (e.g., black or photographic neutral gray) backdrop arranged to reveal diagnostic characteristics of the fungi.
- k After return from the field, macrochemical tests shall be performed on both whole and sectioned specimens according to the tentative identification to genus (6.2.1e).

Note: Specification of macrochemical tests valuable used for particular genera exceeds the scope of this Plan. Examples are reactions of context regions or surfaces of boletes to 10%  $\text{NH}_4\text{OH}$ , reaction of the pileipellis of white amanitas to 10% KOH, reaction of context of half longitudinal section of entire sporocarp to paracresol (specific for tyrosinase) and syringaldazine (specific for laccase) solution (Marr, 1979).

## **6.2.2 Collecting Fleishy Fungi**

### **Requirements**

- a The term “fleshy fungi” shall be construed to include mushroom-type fungi, Discomycetes, and fleshy (non-perennial) polypores.
- b Prior to collection, color photographs of fleshy fungi shall be taken in situ.

- c All sporocarps of fleshy fungi with geotropism of lamellae or stipe shall be transported in and from the field in their wrappers (6.2.1a, 6.2.1c) so as to keep the stipe and gills oriented vertically.

Note: This is necessary in order for ease of sectioning, ease of photography of sectioned specimens, and for a sufficiently thick spore print to be obtained in several taxa (e.g., species of *Amanita*).

### **6.2.3 Collecting Fungi with Perennial Sporocarps**

#### **Requirements**

- a The term “perennial sporocarp” shall refer to those fruiting bodies that are relatively long lasting, tough to leathery in texture, and do not become greatly distorted upon drying.
- b A representative sample covering all morphological variation of the sporocarps shall be collected.
- c Variation in sporocarp size shall be recorded.
- d Notes on rot type (e.g., white rot or brown rot) and condition of the host tree or substrate (6.2.1g) shall be recorded for each collected specimen.
- e Rhizomorphs, sclerotia and other sterile structures associated with a collected sporocarp shall be gathered and included with the sporocarp in a single collection.
- f Preparatory to drying, large, perennial polypores
  - i They shall be cut into thin pieces each containing a portion of the pileus surface, context, tubes, and pore surface and sized to fit in the largest herbarium boxes or packets.
  - ii Such sections shall represent the variability seen in the specimens.
  - iii Notes on the entire sporocarp shall be maintained with the conserved portion of the specimen.

## **6.3 Protocols for Collecting and Processing Microfungi**

### **6.3.1 Collecting and Processing Microfungi**

#### **Requirements**

- a When sporocarps (e.g., ascomata and conidiomata) are located,
  - i their maturity shall be determined, by careful observation and dissection with the aid of hand lens or dissecting microscope
  - ii they shall be collected/retained only if they appear mature, but not over-mature.

- b In the field, fragile sporocarps including those with upright structures such as synnematus imperfect fungi with a suitably sized attached portion of substrate shall be mounted with glue in match boxes or other boxes of appropriate size to preserve delicate structures and dried later within their boxes.
- c Slides shall be prepared using the double cover-glass method (7.4.1), but with diagnostic material placed in water and without the introduction of glycerin or final sealing of the slide until mature spores have been measured.
- d Using such slides (6.3.1c) microscopic observations shall be made as quickly as possible including photomicrography of ephemeral structures such as ascus pore plugs, iodine reactions, and gelatinous ascospore sheaths; and these observations shall be recorded.
- e If a slide (6.3.1c) will be needed for further use, the introduction of glycerin and final sealing of the slide per the double cover-glass procedure (7.4.1) shall be carried out.

## **6.4 Protocols for Collecting and Processing Myxomycetes**

### **6.4.1 Collecting and Processing Myxomycetes**

#### **Requirements**

- a Sporocarps shall be collected carefully with substrate attached.
- b In the first year of the ACG ATBI, all sporocarps shall be collected and a list of the most common easily recognizable Myxomycete taxa of each sampling unit shall be developed.
- c In the second through seventh year of the ACG ATBI, when collecting in a given sampling unit,
  - i taxa on the list of most common and easily recognizable taxa for that sampling unit (6.4.1b) shall be recorded when encountered, but not collected
  - ii taxa not on that sampling unit's list of most common taxa (6.4.1b) shall be collected when encountered.
- d In the field, sporocarps with a suitably sized attached portion of substrate shall be mounted with glue in match boxes or other boxes of appropriate size to preserve delicate structures and dried later within their boxes.
- e In laboratory processing, laboratory parataxonomists shall
  - i check material sorted into taxa by the field parataxonomists to reduce mixing of taxa in the same specimen

- ii give preliminary identifications (e.g., to major group, family, genus, or species, when possible).
- f In the laboratory, standardized descriptive notes shall be taken for each collection based on the characters enumerated by Farr (1981: 19-24).
- g Photographs shall be taken of each collection against a black or photographic neutral gray background.
- h Cultures shall be made if needed for identification or to obtain fungal germplasm for other purposes.

## 6.5 Procedures for Post-Collection Processing by Curators

### 6.5.1 Labeling and Packeting of Dried Collections

#### Requirements

- a Specimens of each species collected as part of the Fungus ATBI shall be deposited in a reference collection (herbarium) to document the occurrence of that species.
- b The collection of fungus specimens shall be managed and curated in a manner similar to that of an herbarium of plants (Forman and Bridson, 1989).
- c Initial labeling of specimens of macromycetes and Myxomycetes shall include sampling unit/plot data, date, tentative field identifications, full field notes, and reference to the location of any photographs taken of the collection.
- d Unique accession numbers shall be added by curators during processing.
- e A data base shall be developed using the unique reference numbers to
  - i support maintenance, organization, and tracking of information associated with each collection
  - ii to be a repository for data necessary to generate labels for all collections.

Note: For some small, delicate, boxed specimens, it may be necessary to print labels with only minimal information to be affixed to the outside of the box while complete labels shall be otherwise stored with the collection (e.g., in a standard herbarium packet enclosing the box).

- f Collections shall be packeted or boxed with an initial label attached to, or included in, the packet or box.
- g Standard herbarium packets shall be made from 100% cotton paper (8.5 × 11 inches) folded twice on each side.

- h Specimens too large for standard herbarium packets shall be placed in boxes of the appropriate size.
- i Labels shall be attached to the flap of a herbarium packet or to the top of a box with water-based, white glue.
- j Labels shall be revised in accordance with identifications by subject matter experts.
- k Old labels shall be maintained in collection packets to provide a history of the taxonomic treatment of the collections.

### **6.5.2 Filing of Dried Collections**

#### **Requirements**

- a One or more packets of a single fungal species on a common host shall be glued to an herbarium sheet using water-based white glue.
- b These herbarium sheets shall be standard vascular plant sheets made of relatively stiff paper 11 x 17 inches, equal in size to the length and width of a shelf in an herbarium case.
- c Each herbarium sheet shall be labelled in the lower, right hand corner with the names of the relevant fungal species and host to allow quick perusal of specimens.
- d Herbarium sheets upon which specimen packets are mounted shall be grouped in genus folders of stiff paper that are labelled with the contents.
- e Genus folders with sheets of specimens shall be labelled and placed in herbarium shelves.
- f Genus folders shall be filed according to major taxonomic groups arranged alphabetically by genus.
- g Specimens in labelled boxes shall be placed in large herbarium boxes that match the dimensions of one herbarium shelf.

### **6.5.3 Long-term Preservation of Collections**

#### **Requirements**

- a Collections shall be frozen at -20 deg. C for three days for insect control.

Note: The step is essential for preventing infestation of dermestid beetles which are particularly prone to destruction of large sporocarps such as those of mushrooms and polypores.

- b A regular schedule of refreezing all collections in the herbarium shall be established.

- c Curators and the Herbarium Manager (3.3.1e) shall be responsible for coordinating with subject matter experts to maximize the number of taxa determined in the available time.

## **6.5.4 Limitations on Number of Collections to be Retained**

### **Requirements**

- a Collections sufficient to document the variability of each inventoried species shall be placed in the reference collection.

Note: The number of specimens of a species to be deposited depends on the taxonomic knowledge about that species. More specimens are needed if the identity and circumscription of a species is not well-defined.

- b The number of collections per species from one inventory site shall be between five and 30—not including specimens that are sent as gifts to taxonomic experts for identification.

## **6.6 Sampling-Related Computations Mentioned in the Plan**

### **6.6.1 Computation of Overlap**

#### **Requirements**

- a Given two lists of species (e.g., lists of macromycetes present in two sampling units), the overlap of those two lists shall be computed by the formula  $\frac{a}{a + b + c}$  in which  $a$  is the number of species shared between the two lists;  $b$  is the number of species in the first list and not in the second; and  $c$  is the number of species in the second list and not in the first.
- b The value of the above formula shall be multiplied by 100 and presented as a percentage.

# Common Protocols for Isolation and Culturing Fungi and Managing a Culture Collection

## 7.1 Protocol for Surface Sterilization

### Requirements

- a Surface sterilization of sample materials shall be done by one of the two following methods:
  - i in the case of non-woody plant parts, by use of an alcohol-chlorine bleach-alcohol series (with time in each solution varied according to the thickness of the tissue being sterilized and proportional to the following: 1 minute in 75% alcohol, 3 minutes in 3% chlorine bleach, 30 seconds in 75% alcohol) and then washing in sterile water
  - ii in the case of woody plant parts and roots, by immersion in 95% ethanol followed by flaming.

## 7.2 Protocols for Preparation of Media

### Requirements

- a Unless otherwise noted in a given recipe, media shall be autoclaved at 121 deg. C for 15 min.
- b The following recipes shall be used in the preparation of the most common culture media:
  - i *Benomyl Dichloran Streptomycin (BDS) Agar*. Ingredients include 15 g malt extract, 15 g agar, 10 ml stock solution, 100 mg streptomycin, and 1000 ml of distilled water. The stock solution is composed of 40 mg benomyl 50% w.p. in 50 ml warm 95% ethanol. Dilute stock solution to 100 ml with water and add 20 mg dichloran (2,6-dichloro-4-nitroaniline). Streptomycin is added to the medium after autoclaving.
  - ii *Carboxymethylcellulose (CMC) Agar*. Ingredients include carboxymethylcellulose (15 g),  $\text{NH}_4\text{NO}_3$  (1 g),  $\text{KH}_2\text{PO}_4$  (1 g),  $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$  (0.5 g), yeast extract (1 g), agar (20 g), and 1000 ml of distilled water. The agar is added after the other ingredients are dissolved. The medium is then autoclaved.
  - iii *Cornmeal Agar*. Ingredients include yellow cornmeal (40 g), agar (20 g), and distilled water (1000 ml). The water is

brought to a boil, then the cornmeal is added and the mixture simmered 15 min. before being filtered through 3 or 4 layers of cheesecloth into a 1000 ml graduated cylinder and left standing for 15 min. The top 800 ml is then removed by siphon. The volume is brought back to 1000 ml with distilled water; and the agar, added. This final mixture is sterilized by autoclaving.

Note: Commercial preparations of Cornmeal Agar are available and are satisfactory.

- iv *Cornmeal Dextrose Agar*. Ingredients include commercially prepared Cornmeal Agar (17g), dextrose (2 g), yeast extract (1 g), and distilled water (1000 ml). The agar is added after the other ingredients are dissolved. The medium is then autoclaved.
- v *Cornmeal-Malt-Yeast Extract (CMMY) Agar*. Ingredients include commercially prepared Cornmeal Agar (17g), malt (1 g), yeast extract (1 g), and distilled water (1000 ml). The agar is added after the other ingredients are dissolved. The medium is then autoclaved.
- vi *Cornmeal PPM Agar*. Follow the recipe for Cornmeal Agar (7.2b(iii)) and add to 1000 ml of that medium 10 mg pimarin, 10 mg rifampicin, and 250 mg ampicillin.
- vii *Dilute Malt Extract Agar*. Follow the recipe for Malt Extract Agar, but use 5 g of malt extract instead of the quantity listed there (7.2b(ix)).
- viii *DRBC Agar*. Ingredients include peptone (5 g), glucose (10 g),  $\text{KH}_2\text{PO}_4$  (1 g)  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.5 g), dichloran (0.002 g), rose bengal (0.025 g), chloramphenicol (0.1 g), agar (15 g), and 1000 ml distilled water. When the solutes are dissolved, the final pH must be  $5.6 \pm 0.2$ . The medium is then autoclaved.
- ix *Malt Extract Agar*. Ingredients include malt extract (25 g), agar (15 g), and deionized water (1000 ml). When the malt extract is dissolved, add the agar and autoclave.
- x *Malt-Yeast Agar*. Ingredients include malt extract (5 g), yeast extract (1 g), agar (15 g), and 1000 ml deionized water. Dissolve malt and yeast extracts in water, then add agar. The medium is then autoclaved.

Note: Recommended for isolating saprophytic fungi.

- xi *Malt-Yeast-Peptone Agar*. Ingredients include malt extract (6 g), yeast extract (0.5 g), peptone (1 g), agar (20 g), and 1000 ml deionized water. Dissolve malt and yeast extracts and peptone in water, then add agar. The medium is then autoclaved.
- xii *Oatmeal Agar*. Ingredients include rolled oats (30 g), agar (20 g), and 1000 ml of deionized water. The oatmeal is cooked in the water for 15 to 30 min. in a container over boiling water (e.g., a double boiler may be used). The cooked oatmeal shall be filtered through 3 or 4 layers of cheesecloth; and the filtrate, brought back to 1000 ml with distilled water. The agar is then added, followed by autoclaving.

Note: Commercial preparations of Oatmeal Agar are available.

- xiii *Potato Dextrose Agar (PDA), Commercial*. Ingredients are PDA (39 g) and 1000 ml of distilled water. The PDA is dissolved in the water. The medium is then autoclaved.
  - xiv *Potato Carrot Agar*. Ingredients include potatoes (300 g, sliced with skin on), carrots (25 g, peeled and sliced), agar (15 g), and 1000 ml tap water. The potatoes and carrots are boiled in the tap water and simmered for 20 minutes. The cooked vegetables are filtered out through 3 or 4 layers of cheesecloth. The filtrate is returned to 1000 ml with distilled water. The agar is added and dissolved. For half-strength Potato Carrot agar, bring the filtrate to 2 liters after filtering and prior to addition of agar and use 30 g of agar. Sterilization is by autoclaving at 121 deg. C for 20 min.
  - xv *Tap Water Agar*. Ingredients include agar (20 g) and 1000 ml tap water. The agar is dissolved in the water. Autoclave.
- c The media for growing *Dictyostelium* shall be
- i composed of 0.5 g glucose, 0.5 g peptone, 25 g agar dissolved in 1000 ml water
  - ii autoclaved
  - iii prepared 6 or 7 days prior to expected use
  - iv streaked with nutrient broth after cooling and just before dictyostelid spores are added.
  - v use nutrient broth containing living bacteria (*E. coli*) by placing one or two drops of this broth on the plate and tilting the plate back and forth to spread the broth.

Note: The bacterial suspension need not be very dense even if the agar were low in nutrients. It takes several hours to a day or more for spores of dictyostelids to germinate into amoebae. The bacteria will start to grow and spread well before the dictyostelid spores germinate (John Landolt, pers. comm.).

- d Prior to their use, media shall be stored under refrigeration in stacks or racks, but not in plastic sleeves.

Note: Storage in plastic sleeves has been noted to result in increased contamination.

## 7.3 Protocols for Isolation and Culturing Techniques

### 7.3.1 Protocols for Intensive Isolation of Fungi from Pulverized and Washed Samples

#### Requirements

- a All media used to plate out samples (i.e., those pulverized samples covered by 8.5.2, 9.1.3, 10.1.6e(iv), and 10.1.7) shall contain at least two antibiotics—e.g., streptomycin sulfate and chlortetracycline hydrochloride at 50 mg/L—unless option 7.3.1o is exercised.
- b At the same time that isolation media are prepared, agar slants shall be prepared for transfer, development, and sorting of isolates.
- c For each sample, the following supplies shall be prepared:
  - i 500-1,000 agar slants such as Malt Extract Agar (7.2b(ix)), Malt-Yeast Agar (7.2b(x)), PDA (7.2b(xiii)), and Corn Meal Agar (7.2b(iii)) for storage

Note: The slants are to be used for storage of cultures.

- ii 100-200, 60 mm plates with assorted media such as Cornmeal Agar (7.2b(iii)), Oatmeal Agar (7.2b(xii)) or Malt Extract Agar (7.2b(ix)) for subculture and identification.
- d During early stages of incubation, all fast-growing colonies shall be eliminated from plates as quickly as possible.

Note: Removal of fast-growing fungi permits development of slower growing species, thus amplifying the diversity of isolates.

- e From each sample, isolation shall be carried out by all the following methods:
  - i Isolation by particle filtration (7.3.1f)
  - ii Warcup soil plates (7.3.1g)
  - iii ethanol pasteurization method (7.3.1h)
  - iv horse hair bait method (7.3.1i)

- v methods specific to Oomycetes (7.3.1j)
  - vi methods specific to chytrids (7.3.1k)
  - vii methods specific to dictyostelids (7.3.1l)
  - viii methods specific to yeasts (7.3.1m)
  - ix methods specific to soil Basidiomycetes (7.3.1n).
- f Isolation by particle filtration (soil washing) shall be accomplished as follows:
- i A finely pulverized sample shall be washed with a stream of sterile distilled water and filtered by this series of filters: a brass 2 mm prescreen, a sterilized polypropylene 210  $\mu\text{m}$  mesh, and a sterilized polypropylene 105  $\mu\text{m}$  mesh.
  - ii Approximately 1 ml of particles shall be collected.
  - iii These particles (7.3.1f(ii)) shall be rewashed 3 times by being suspended by agitation in 1:20 volume/volume of sterile water, being allowed to settle, and having the water poured off.
  - iv One twentieth of the rewashed particles (7.3.1f(iii)) suspended in 0.1 ml of sterile water shall be applied to each of 20 plates comprising equal numbers of plates prepared with 4 different media including: Carboxymethylcellulose Agar (7.2b(ii)), Cornmeal Dextrose Agar (7.2b(iv)), Dilute Malt Extract Agar (7.2b(vii)) with Cyclosporin<sup>TM</sup> A<sup>1</sup>, and other soil isolation media appropriate for ascomycetous and mucoraceous fungi.
  - v The 20 plates (7.3.1f(iv)) shall be incubated at 25 deg. C with a 12 hour photoperiod and checked every two days for one month.
- g Isolation via Warcup soil plates shall be carried out as follows:
- i One or two plates shall be established per sample.
  - ii Fine particles of the pulverized sample (1 mg) shall be placed in the bottom of dishes by means of a spatula.
  - iii Media shall be poured into these dishes (7.3.1g(ii)) and shall be agitated to disperse the particles.
  - iv Plates prepared according to the Warcup soil plate method (7.3.1g(ii)) shall be incubated at 25 deg. C with a 12 hour photo period.

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<sup>1</sup>Cyclosporin is a trademark of Novartis.

- v Fungal colonies developing in these plates (7.3.1g(iv)) shall be excised and plated to determine identity.
- vi Plates (7.3.1g(v)) shall be further observed until no additional species appear on them.
- h The ethanol pasteurization method shall be carried out as follows:  
 Note: This method kills fungi with thin-walled hyphae and conidia, thus selecting for species with resistant ascospores, conidia or sclerotia.
  - i 20 - 50 mg dry, finely pulverized sample shall be placed in a sterile tube.
  - ii This sample (7.3.1h(i)) shall be immersed in 60% ethanol for 4 minutes and then the liquid decanted.
  - iii The treated sample (7.3.1h(ii)) shall be washed immediately with a small quantity of sterile water into 125 ml of molten Malt Extract Agar (7.2b(ix)) with Cyclosporin A or DRBC agar (7.2b(viii)) to yield 5 plates of each medium per sample.
  - iv Plates prepared according to the ethanol pasteurization method shall be incubated between 15 deg. C and 25 deg. C.
- i Horse hair bait to recover keratinophilic fungi occurring in soil shall be used in the following manner:
  - i Moistened soil shall be placed in deep dish plates.
  - ii A few paradichlorobenzene crystals shall be added to kill mites.
 Note: Paradichlorobenzene is carcinogenic and should be handled according to pre-established safety procedures.
  - iii Autoclaved horse hair shall be placed on top of the soil (7.3.1i(i)).
  - iv Two to four plates shall be prepared per sample.
  - v The plates shall be incubated between 20 deg. C and 25 deg. C and observed for up to 2 months.
- j The method for isolating Oomycetes shall be as follows:
  - i Samples shall be plated on selective media (e.g., Cornmeal PPM Agar [7.2b(vi)]) containing fungicides toxic to true fungi.
 Note: This will allow differential growth of protocistan Oomycetes.
  - ii Organic baits (e.g., seeds, insects, snake skin, etc.) shall be incubated with pulverized samples suspended in sterile water to capture particular groups.

- iii After incubation for about one week, these baits (7.3.1j(ii)) shall be examined for development of sporulating structures.
- k The method for isolating chytrids shall be as follows:
  - i Organic baits, e.g. pollen, shall be incubated in pulverized samples suspended in distilled water.
  - ii After incubation for about one week, these baits (7.3.1k(i)) shall be examined for development of sporulating structures.
- l The method for isolating dictyostelids shall be as follows:
  - i A pulverized sample shall be plated on selective media (prepared as described in 7.2c) containing bacterial baits.
  - ii Spores from mature sporocarps shall be taken by transfer needle and placed in the center of the plate containing the medium.
  - iii The methods shall be those of Cavender and Raper (1965).

Note: After three to four days at room temperature, one can observe simultaneously mature sporocarps near the center of the plate, aggregations further out, and vegetative amoebae near the periphery of the plate (John Landolt, pers. comm.).

- m The method for isolating yeasts shall be as follows:
  - i Pulverized samples shall be added to acidified media, e.g. pH 3.7 using N hydrochloric acid.
  - ii The resulting mixture (7.3.1m(i)) shall be plated on an isolation medium.

Note: Yeasts are recovered at high frequency on specific isolation media.

- n The method for isolating Basidiomycetes shall be as follows:
  - i Saprobic Basidiomycetes shall be selectively isolated on Malt Extract Agar (7.2b(ix)) media with benomyl (4 mg/L), guaiacol (0.4 ml/L) and lignin (in the form of indulin AT at 1 g/L) components (Thorn et al., 1996).
  - ii Ectomycorrhizal Basidiomycetes shall be sampled on the basis of their fruiting bodies only and not cultured.

## Options

- o Antibacterials not listed in 7.3.1a, such as chloramphenicol, may be substituted or added as a supplement in the media.

### **7.3.2 Protocol for Study and Preservation of Cultures**

#### **Requirements**

- a The morphology of cultures shall be fully described using both dissecting and compound microscopes.
- b The descriptions of culture morphology shall be databased in a structure that
  - i provides accessibility by character(s), taxon, culture identifier, collection data, etc. similar to the structure used for herbarium specimens
  - ii provides accessibility by conditions of isolation.
- c Descriptions of cultures should include growth rate, appearance, texture, smell, and other characteristics of the colony, chemical reactions, and morphological characteristics. Cultures must be examined during the early stages of sporulation in order to observe conidial development.
- d All cultures shall be preserved in the INBio culture collection.

### **7.4 Protocols for Preparation and Curation of Permanent Microscope Slides**

#### **7.4.1 Protocol for Making Permanent Slides**

##### **Requirements**

- a All permanent slides shall be made by the double cover-glass method (Kohlmeyer and Kohlmeyer, 1972, 1979; Volkmann-Kohlmeyer and Kohlmeyer, 1996).

#### **7.4.2 Labeling and Post-Collection Processing of Permanent Slides of Micromycetes by Curators**

##### **Requirements**

- a All permanent slides shall be marked with identification of taxon, unique collection or culture identifier, and date of preparation.
- b It shall be possible to correlate each slide with the original specimen from which the slide was made and other parts of the same collection, any descriptive notes on the slide, and any drawing or photomicrographs of the material on the slide.
- c It shall be possible to access easily the material (7.4.1b) related to a slide.

## 7.5 Protocols for Managing Culture Collection

Methods for the long-term storage of living fungal cultures vary with the organisms to be stored and the resources available. The most reliable and maintenance-free preservation is by freeze-drying (lyophilization) and storage in liquid nitrogen. Both these techniques require specialized equipment. It is expected that the Fungus ATBI will share lyophilization equipment and relevant staff with other ATBIs (Fig. 1), but liquid nitrogen may not be available in sufficient supply in Costa Rica. Alternative techniques include serial transfer on low nutrient agar, storage in mineral oil or sterile water or dried in soil or silica gel. Advantages of different techniques for different fungal groups are delineated in Smith and Onions (1994); but it should be noted that some of the tropical Basidiomycetes may not survive after being stored in liquid nitrogen, or, sometimes, even at 5 deg. C (D. J. Lodge, pers. comm.).

### Requirements

- a Initially, all living cultures shall be stored on Cornmeal Agar (7.2b(iii)) slants in a refrigerator (i.e., at 4 deg. C) unless it is known that this medium and manner of storage are inappropriate.

Note: Most fungi can be stored as above. Exceptions include Oomycetes, Entomophthorales, and some wood decaying Basidiomycetes. In general, mycorrhizal Basidiomycetes will not be cultured (7.3.1n(ii)).

Note: A screw-top tube is the most convenient container for these cultures, although glass test tubes with cotton plugs also can be used.

- b Cultures of wood decaying Basidiomycetes shall be stored on Malt Extract Agar (7.2b(ix)) slants in a refrigerator.
- c Cultures of Oomycetes and Entomophthorales shall be stored under sterile water at room temperature.
- d Each stored vial (7.5a) shall be marked with a unique accession number that shall facilitate access to all information concerning the culture in that vial in an electronic data base.
- e Storage records shall be maintained for each fungal culture in long-term storage; and maintained data shall include identity; origin with such details as unique culture and other relevant accession numbers, site, substrate, method of isolation; method of preservation; and last date of transfer.

Note: A unique culture number or accession number can be used to link the culture or specimen with items associated with that culture/specimen such as photographs, notes, permanent slides, uncultured parts of a collection.

### **Options**

- f For those cultures that sporulate readily, storage may be by freeze-drying when necessary (e.g., due to limited refrigeration resources).

## Sampling Protocols—Fungi Associated with Living Plants and Fungicolous Fungi

Fungi associated with living plants and plant parts range from obligate biotrophs to facultative, symbiotic generalists. Many of these fungi are plant pathogenic causing serious diseases of agricultural, horticultural, and native plants; others are beneficial, for example, protecting the plant from insect predation, or neutral, apparently existing without harm or benefit to the host. For at least part of their life cycle, many of the fungi associated with living plants are host-specific, usually at the level of plant species, genus or group of closely related genera. Among these are Ascomycetes such as Erysiphales (powdery mildews), Meliolales (black mildews), Taphrinales and Phyllachorales; Basidiomycetes such as Exobasidiales, Ustilaginales (smuts), and Uredinales (rusts); and some protist taxa especially the Peronosporales (downy mildews).

Endophytic fungi that appear to have no effect on the host are often associated with the internal tissues of living plants. Recent studies have shown that most living plants harbor a consortium of organisms spanning diverse taxonomic groups. These consortia are often specific to a particular host and may yield many previously unknown fungal species. Less conspicuous plants such as mosses and algae as well as living fungi including lichens are often infected with such microfungi.

The majority of endophytic fungi seem to exist in a more or less latent state within the living plant tissue, but once the host tissue becomes weakened or dies due to some cause other than the fungal infection, the endophytic fungi rapidly convert either to saprobic nutrition or necrotrophy, killing the plant part. Some endophytic fungi occupy living tissue for their entire life cycle, perpetuating themselves between plant generations by infecting ovules or other plant parts. Some endophytic fungi are known to have mutualistic relationships with their plant partners such as the grass endophytic fungi that avert insect predation. Other endophytes have no apparent affect on their host plants.

The border between living and dead plants is not always distinct, for living plants may include dead tissues such as the bark of woody trees. Although technically not living, plants parts that are still attached will be sampled as part of the sampling of fungi on living plants.

Entomopathogenic fungi attacking insects living on plants will also be collected during the sampling of fungi on living plants.

## 8.1 Substrates

Fungi inhabit all kinds of plant parts; and each plant part may support a diverse array of fungi. These plant parts are summarized as follows:

- a) *Living Leaves, Twigs, and Stems.* Fungi to be found on these substrates include biotrophic fungi immersed and erumpent from green tissue; necrotrophic fungi causing lesions, spots, and other symptoms; saprobic secondary colonizers of dead tissue killed by necrotrophs; superficial biotrophic fungi such as sooty molds having haustorial infection of living tissue; superficial saprobic fungi, deriving nutrition from honeydew and other exudates; foliicolous lichens; yeasts and other phylloplane fungi; endophytes of specific leaf parts such as laminae, vein, midribs, petioles, and entomopathogenic fungi attacking plant inhabiting insects.

Note: Sampling protocols for entomopathogenic fungi are among those specified in chapter 11.

- b) *Dead, Attached Leaves and Twigs and Plant Parts Including Petioles, Seeds, and Persistent Fruits.* Saprobic phases of biotrophic, necrotrophic and endophytic fungi; secondary colonizers may occur on these substrates; some, but not all, of the fungi on these substrates also will be found on terrestrial substrates.
- c) *Flowers.* Necrotrophic fungi may occur on petals and sepals, especially of large, fleshy or long-lived flowers; yeasts are associated with nectar.
- d) *Fruits.* Biotrophic and necrotrophic fungi may occur on developing and mature fleshy and persistent fruits; yeasts and other saprobic fungi may appear as secondary colonizers.
- e) *Specialized Organs such as Extrafloral Nectaries, Spines, Lianas.* Biotrophic and necrotrophic fungi may occur on these organs; however, these structures have generally not been examined for associated fungi.
- f) *Bark and Wood Including Slime Fluxes.* To be expected on these substrates are endophytes of wood and bark; biotrophic and necrotrophic fungi on bark; lichenized fungi; saprobic secondary colonizers; saprobic yeasts and filamentous fungi on slime fluxes.

- g) *Roots*. Parasitic and gall-forming taxa such as the Plasmodiophorales, endophytic fungi, ecto- and endomycorrhizal taxa are associated with roots.
- h) *Fungicolous Fungi*. Biotrophs, necrotrophs and secondary saprobes may be found on all fungi, including lichens.

## **8.2 Host Specificity**

The host specificity of many fungi associated with living plants is unknown, and obtaining such information during the Fungus ATBI will be valuable. Some generalizations can be made about the host specificity of fungal groups inhabiting living plants, based on the presumed nutritional status of the fungal group. Sampling should be concentrated on habitats and hosts that harbor fungal groups with narrow host ranges. Sampling for fungal taxa with wider host ranges can be carried out at the same time, but progressively abandoned at later stages of the Fungus ATBI as the same fungal species are encountered repeatedly.

## **8.3 Introduction to Sampling Protocols for Fungi Associated with Living Plants and Fungicolous Fungi**

Inventorying for fungi associated with living plants can be undertaken using two approaches, namely by direct observation of fungal species on the surfaces of living leaves and bark and by indirect observation through the isolation of fungi from living substrates. Because many of these fungi are host-specific or of unknown host-specificity, sampling for fungi by direct observation from specific plant taxa clearly requires collection primarily on the basis of host plant species. Location of plant species by those working on the Plant ATBI will therefore be the first step for most sampling.

Collection of fungi associated with plant hosts could be carried out by those working on the Plant ATBI. It would be advantageous to instruct plant collectors to retain plant samples with fungal lesions or other symptoms, and pass them on to the Fungus TWIG. This is particularly important for those plant species that are rare or difficult to collect or identify.

## **8.4 Sampling Protocols for Fungi Associated with Living Plants and Fungicolous Fungi by Direct Observation**

### **8.4.1 Sampling Fungi Associated with Living Plants and Fungicolous Fungi by Direct Observation—Common Protocols**

#### **Requirements**

Note: Macromycetes are to be collected and processed in accord with 6.1 and 6.2. Myxomycetes are to be collected and processed in accord with 6.1, 6.4 and 6.5. Lichenized fungi are to be collected and processed in accord with 6.1.

- a At the beginning of the Fungus ATBI, samples shall be taken in and around established sampling units (5.1).

Note: A suite of relatively common species can be sampled without following protocols for sampling specific hosts.

- b After the Plant TWIG has produced a critical mass of data on plant distributions, specific host species shall be targeted using a combination of direct assistance from the Plant TWIG members and GPS data.
- c Once the target hosts are determined, populations of host taxa shall be located and recorded as Fungus ATBI collecting sites.

Note: Target hosts will represent at least two genera in each plant family (9.1.2b).

- d At the established collecting sites, an intensive sampling of all parts of the host plants shall be undertaken.
- e Long-lived host plants (or areas containing populations of short-lived plants) from which collections are made shall be marked by a means that will last at least for the duration of the Fungus ATBI.
- f Voucher specimens of all hosts shall be collected in order to accurately identify these plants.
- g Names and other information concerning plants on which fungi are not found shall also be recorded.
- h As plants in the Fungus ATBI plots are sampled for fungi, records of unsuccessful searches shall be the basis for stop rules (3.3.2a, 3.3.2b).

#### **Recommendations**

- i Plant ATBI collectors should be instructed to retain plant samples with fungal lesions or other symptoms, and pass them on to the Fungus TWIG.
- j At least five widely separated populations of each host plant species should be sampled when possible.

- k An attempt should be made to sample representative species of each host genus.

Note: For bryophytes and algae on which very small fungi—those with sporocarps less than 100  $\mu\text{m}$  diam—are known to exist, the logistics of sampling each species may be insurmountable in the seven year period of the Fungus ATBI.

- l Collection of fungicolous fungi, i.e., those deriving nutrition from other fungi in mutualistic, antagonistic, or saprobic relationships, should be carried out in tandem with sampling of their host species collected on nonfungal substrates.
- m For fungicolous fungi, repeated sampling of the host fungus shall be done at various stages in the host's life cycle.

#### **8.4.2 Direct Observation of Fungi on Living Plant Parts Except Bark**

##### **Requirements**

- a For herbaceous plants, shrubs, small trees, palms, and ferns having green parts within reach of the ground, at least 200 individuals (in total for all sites) shall be searched every two months for a period of one year.

Note: Sampling fungi on living leaves of deciduous plants and on ephemeral plants will be limited by substrate presence. This difficulty may be partially overcome by close interaction with those working on the Plant TWIG.

Note: For large trees and for most of their epiphytes it will be necessary either to fell the sample trees or to remove large branches before they can be searched as discussed under the section on wood-associated fungi (9.1.2 through 9.1.4). Coordination with other TWIGs needing access to the canopy would be advantageous; frequency of sampling fungi in the canopy will necessarily be limited. However, the high number of leaves and branches of large trees in part makes up for sampling fewer individuals.

- b Field parataxonomists shall be trained to observe fungi restricted to various niches and microhabitats (8.4.2c) in order to obtain a thorough sampling of species diversity.

Note: Mycologists refer to this as “developing an eye” for a particular group of fungi in a specific microhabitat. Searching for microfungi on living plant parts requires considerable initiative and independence.

- c Microhabitats shall be located by
  - i sampling at different compass points around tree trunks
  - ii sampling leaves and other plant parts of various ages

- iii sampling substrates growing in diverse conditions of light intensity and soil moisture
- iv sampling at increasing levels on specified tree trunks from ground level to the canopy.
- d When making a collection, biotic and abiotic data shall be recorded including vegetation type, altitude, light intensity, and a gross estimate of moisture content of the substrate.
- e Where possible, age of each sampled plant part shall be recorded.
- f Plant parts with recognizable signs or symptoms of fungal presence shall be sampled; and, if possible,
  - i such samples shall include five separate collections each from separate plants in the same locality
  - ii such samples shall include between 10 and 20 leaves for those plants having leaves more than 10 cm long or having leaves on which few fungi are found and 50-100 leaves for plants having small leaves or perennial leaves with large numbers of fungi.

Note: When leaves have a large number of different fungi on them, as can be the case for tropical trees with perennial leaves, the sample size ought to be near the larger end of the suggested range. Samples ought to be toward the smaller end of the range if suitable leaves infected with fungi are not found.

- g Special attention shall be paid to collecting from diseased plant parts bearing deformed lobes, black spots, blisters, and galls particularly in areas where the host species are common.
- h Leaves and leaf clusters shall be removed from plants and placed in paper bags or packets with the exception that plastic bags may be used if necessary due to rainfall.

Note: Sufficient voucher material to identify the plant must also be included if on-site identification is not feasible (8.4.1f).

- i In the laboratory, samples shall be briefly examined by a curator in order to decide whether incubation or culturing is desirable.

Note: Because many taxa are difficult to culture apart from their hosts, fungi that are obligately associated with living plant parts will not be routinely cultured. However, culturing of some fungal species is necessary to establish their identity and allow for description of the vegetative phase of the life cycle.

- j Overmature biotrophic fungi shall be sought again and recollected after an appropriate interval to obtain mature, but not over-mature specimens.
- k If a collected fungus is immature, samples shall be placed in a moist chamber until sporulation occurs.
- l Fungal specimens on living leaves and other small plant parts shall be prepared as herbarium specimens by drying them in plant presses in the manner used for uninfected vascular plants by the Plant ATBI.
- m Representative samples of bryophytes, lichens and algae, including apparently healthy, dying and dead parts shall be brought back to the laboratory for analysis with a dissecting microscope, and possible culturing.

Note: Fungal associates of small herbaceous plants, bryophytes, lichens and algae are almost all very inconspicuous, and are unlikely to be detected during field sampling unless systematic sampling and special consideration is given to these habitats.

### **8.4.3 Direct Observation of Fungi on Living Tree Bark**

The majority of species of bark-inhabiting fungi are not host-specific, although some have affinity for particular kinds of bark. To obtain the host-specific fungi requires extensive sampling. Many fungi on living bark are lichenized having more or less conspicuous thalli, often with several lichen species growing intermixed on the substrate.

#### **Requirements**

- a Sampling by direct observation shall be undertaken for fungi on bark in a manner similar to that described for fungi on living leaves (8.4.2).
- b When living trees are felled to sample the canopy organisms as described in the section about sampling fungi on wood (9.1.2 through 9.1.4), bark-inhabiting fungi shall be included in the sampling process.
- c A complete sampling of bark at or near the base of a tree shall be made for all host species.

Note: This does not depend on felling of trees.

- d For the first two sampling cycles of the six proposed for fungi on leaves (8.4.2a), all bark-inhabiting fungi shall be collected.
- e For the remaining four sampling cycles (8.4.3d), only the inconspicuous fungi, i.e., those lichenized fungi without obvious thalli, shall be included.

Note: This reduces duplicate sampling. Some repetitious collection of lichenized taxa with inconspicuous thalli will occur.

- f With optional exception of large foliose lichens (8.4.3l), the sampling procedure shall consist of cutting out pieces of bark with intact, lichenized or non-lichenized fungi present on the bark surface using a stout knife, small axe, folding saw, or secateurs as appropriate.
- g Samples shall be large enough to include a portion of surrounding bark in order to ensure that inconspicuous thalli are collected except in conditions described in option 8.4.3l.
- h If a thallus or stroma of a lichenized fungus to be sampled is too large to fit into a standard specimen packet, portions of that fungus shall be collected that include the edge and central parts of the fruiting structure or thallus.
- i Information shall be recorded including specific location on the trunk (meters above ground), compass point, and tree biometrics including height, diameter-at-breast-height (DBH), height of first branches, and roughness of the bark particularly for foliose lichens when removed without substrate.
- j Collections of fungi from tree bark shall be placed in paper bags and air dried as soon as possible (6.1.2a) except under the conditions described in option 8.4.3m.
- k From each living tree sampled, 15 - 30 square cm of bark without obvious presence of fungi shall be collected for moist chamber culturing for *Myxomycetes* (8.4.4).

### **Options**

- l Large foliose lichens may be removed without the substrate.
- m In humid situations, plastic bags may be used for transport of fungi from tree bark to the laboratory or field dryer, but only for a few hours and when absolutely necessary.

## **8.4.4 Direct Observation of *Myxomycetes* from Bark in Moist Chambers**

### **Requirements**

- a From bark samples of living trees taken for the purpose (8.4.3k), pieces consisting of about 1.5 cm square of surface area and adjacent tissue shall be soaked with sterile water and arrayed on filter paper soaked with sterile water in Petri dishes labeled to indicate the location, tree, date, and other data of origin.

- b Each Petri dish shall be checked for Myxomycetes (especially for small, single fruiting bodies and, if at all possible, without removing the lid) 24 and 48 hours after incubation is established and, then, every two days for the first two weeks and every three or four days thereafter for a period of up to two months.
- c Water shall be added to prevent the contents of the Petri dishes from drying out.

Note: If it is necessary or useful to maintain plasmodia for any length of time, they can be fed oat flakes (Camp, 1937).

- d If a myxomycete fruiting body is found to be developing when a Petri dish is checked, it should be harvested and processed by the methods defined by 6.4.1 and 6.5.1.

Note: It is expected that this method also will yield many taxa of corticolous Hyphomycetes that can be processed according to protocols in 8.5.2.

## **8.5 Sampling Protocols for Fungi Associated with Living Plants and Fungicolous Fungi by Indirect Observation**

Note: All cultures are to be preserved in the INBio culture collection (7.3.2).

### **8.5.1 Indirect Observation of Necrotrophic Fungi on Living Plants**

#### **Requirements**

- a For fungi without mature sporulating structures, isolation at the colony margin shall be undertaken in an attempt to obtain a culture of the fungus killing the plant tissue.
- b This isolation of necrotrophic fungi shall be done by excision of plant tissue at the edges of the colony where the fungus is actively growing.
- c Leaf portions approximately 1 cm square, if possible, shall be surface sterilized (7.1) to remove saprobic secondary colonizers, plated on a weak medium, and incubated.
- d Some tissue also shall be plated without surface sterilization in order to isolate fungi that are particularly susceptible to sterilization.
- e If hyphae grow from the surface-sterilized material (8.5.1c), the non-surface-sterilized tissue shall be discarded.

Note: Most likely, the nonsurface-sterilized tissue to be discarded contains the same fungus as the surface-sterilized material or ubiquitous saprophytes.

- f As they emerge from the sterile leaf portions, fungi shall be transferred to media that induce sporulation and identified.

### 8.5.2 Indirect Observation of Saprophytic Fungi on Dead Leaf Parts and Bark of Living Plants

To identify saprobic fungi on dead leaf parts, culturing is often necessary. The same is true of Hyphomycetes found on bark in moist chambers used for direct observation of other organisms.

#### Requirements

- a Fungal spores or mycelial portions shall be removed from a dead leaf part or bark surface using a dissecting microscope and mounting needle or micromanipulator.
- b These spores or mycelial portions shall be placed on the surface of an agar plate with appropriate media such as half-strength Potato Carrot Agar (7.2b(xiv)) or Tap Water Agar (7.2b(xv)) and incubated until the colonies are mature.
- c Protocols for culturing and identification shall be selected from among those covered in the section on intensive isolation of fungi from pulverized samples (7.3).

Note: Use of particle filtration technique plated on relatively weak media will maximize the number of species isolated.

Note: Using standard culture methods, only filamentous fungi are obtained. For particular groups of organisms, for example, phylloplane yeasts, direct culturing using special techniques and selective media is necessary. In some cases protocols and selective media may need to be developed that favor isolation and culture of specialized groups of fungi.

### 8.5.3 Indirect Observation of Lichenized Fungi on Living Plants

#### Requirements

- a If culturing of a lichen is desired, a specimen of the lichen shall be divided at the time of collection into a portion for an herbarium specimen and another intended for culture, and the material to be cultured shall be brought back to the laboratory quickly.

Note: In general lichens can only be cultured from fresh material, that is, before it has been dried, not from rehydrated specimens.

#### Options

- b Laboratory culture of lichens as separate fungal or algal components may be useful in order to characterize the algal component as part of the Alga TWIG.
- c Laboratory culture of the fungal component of a lichen may be useful in order to characterize that component.

Note: Laboratory culture of lichens as the separate fungal or algal components is not normally required for identification. However, it may be desirable to culture the fungal component following the techniques outlined by Honegger (1996).

### **8.5.4 Isolation of Endophytic Fungi—Common Protocols**

#### **Requirements**

- a Endophytic fungi shall be isolated from excised tissues after surface sterilization (7.1).
- b Initially tissue samples shall be taken from representative host species in 5 genera of each of 11 plant families with diverse life forms, including an aquatic family, a mangrove, two woody and two herbaceous families, a parasite, a succulent, an epiphytic family, a bryophyte family, and a pteridophyte family.
- c In addition, sampling shall take place from all species of two genera from four plant families.

Note: Based on this preliminary study the overlap (6.6.1) in species composition of endophytic fungi among hosts will be evaluated and the level of study needed for further investigations will be determined (8.5.4d).

- d After sampling per 8.5.4b and 8.5.4c for one year of the ACG Fungus ATBI, stop rules (3.3.2a, 3.3.2b) shall be developed to reduce the host list.
- e To carry out indirect observation of endophytic fungi, samples shall be used of leaf, wood and root tissues.
- f Plants with specialized structures such as spines, tendrils, lianas, pneumatophores, glands, galls, and Beltian bodies shall have isolations attempted from all such structures.

#### **Recommendations**

- g The Fabaceae should be included in the inventory of endophytic fungi due to its functional importance in ecosystems.

### **8.5.5 Isolation of Endophytes from Living Leaves**

#### **Requirements**

- a In the field samples shall be collected from five widely separated individuals of each host plant species to be sampled (8.5.4b, 8.5.4c, 8.5.4g).
- b Five leaves or the equivalent shall be taken from each plant, including basal and apical leaves, young and mature leaves, and leaves that grew in sun and those that grew in shade.

- c Samples shall be put in small ice chests (without ice or other coolant) to maintain them at an acceptable humidity and temperature during transport to the laboratory for culturing.
- d In the laboratory, 1 cm square samples of lamina including a vein, midrib, and petiole shall be excised from each leaf.
- e In the laboratory, leaf stems shall be cut into 1 cm lengths.
- f The excised leaf pieces (8.5.5d) and stem pieces (8.5.5e) shall be surface sterilized (7.1).
- g The excised pieces (8.5.5d) and stem pieces (8.5.5e) shall be mixed and a composite of the samples shall be selected.
- h The surface sterilized squares in the composite sample (8.5.5g) shall be cut into approximately 1 mm square pieces; the surface sterilized stem segments in the composite sample shall be cut into 1 mm lengths; and, from these smaller pieces, 20 each shall be selected from lamina samples, and 10 each from other leaf parts.
- i Five of the selected leaf or stem pieces (8.5.5h) shall be placed in Petri dishes on Dilute Malt Extract Agar (7.2b(vii)) amended with antibacterial antibiotics, some with and some without Cyclosporin A.

## 8.5.6 Isolation of Endophytes from Living Wood

### Requirements

- a In the field, two widely separated individuals of each host species to be sampled (8.5.4b, 8.5.4c, 8.5.4g) are selected.
- b From each host individual to be sampled (8.5.4, 8.5.4c, 8.5.4g), sections of trunks or branches 3-5 cm diam and 20 cm long shall be cut and the ends immediately sealed with plastic bags and elastic bands to accelerate the decay process.
- c Each section (8.5.6b) shall be labeled and stored in a safe place out of doors preferably at the site from which it originated.
- d These sections (8.5.6b) shall be examined for fungi immediately and after 6 weeks, 3 months, 6 months, and one year.
- e In addition, using a bandsaw, a disc 2 cm thick shall be cut from each section (8.5.6b) at every sampling time (8.5.6d) and taken back to the laboratory.
- f Each of these discs (8.5.6e) shall be aseptically split in half along a diameter.
- g Working in a laminar flow cabinet, 20 chips about 2 mm (radial)  $\times$  4 mm (longitudinal dimension) shall be extracted from the edges, the

center and the median part of each disc (8.5.6e) using a sterile chisel or knife.

- h These chips shall be plated 10 per Petri dish on Dilute Malt Extract Agar (7.2b(vii)) prepared as defined in 8.5.5i.
- i The sampling of wood endophytes shall be evaluated after 1 year in order to determine if sampling strategies should be modified.

Note: Bark endophytic fungi will not be studied as an independent project. Rather these fungi will be detected as part of the study of wood endophytes or during the successional study of cut logs as outlined in the section on woody substrates (chapter 9).

## **8.5.7 Isolation of Fungi Associated with Roots Other than Mycorrhizae**

### **Requirements**

- a Sampling for non-mycorrhizal root-associated fungi shall occur two to four times per year.
- b Two trees of each representative host species (8.5.4b, 8.5.4c, 8.5.4g) shall be sampled by extracting single 20 cm lengths of 10 woody roots per tree.
- c These root samples (8.5.7b) shall be processed in the same way as other samples of living wood from which endophytic fungi are isolated (8.5.6).
- d Fine roots, i.e., those less than 2 mm diam, shall have 10-20 cm lengths cut from roots of the same host species as those sampled for wood endophytes (8.5.6a).
- e In cloud forest sampling of fine roots (8.5.7d) shall take place in the relatively dry season.
- f In all habitats, sampling of aerial roots shall occur in the wet season.
- g In all cases, sampled roots (8.5.7b, 8.5.7d) shall be traced back to the base of the plant from which they issue in order to provide accurate host determination.
- h Fine root samples (8.5.7d) shall be gently washed in a 0.1% solution of sodium pyrophosphate and examined under a dissecting microscope for the presence of lesions indicating root pathogens, and other evidence of root-associated fungi.
- i Apparently healthy portions of fine root samples (8.5.7d) and zones surrounding lesions on fine root samples (8.5.7h) shall be surface sterilized (7.1) and plated out on media suitable for the isolation of endosymbiotic and phytopathogenic fungi respectively.

Note: Cornmeal-Malt-Yeast Extract Agar (7.2b(v)) or similar weak medium such as CMC Agar (7.2b(ii)) or Dilute Malt Extract Agar (7.2b(vii)) is recommended for isolation of phytopathogenic fungi. Chytridiomycetes and Oomycetes require specialized isolation media as described in Singleton et al. (1992). Most root pathogens are also encountered as free-living fungi in soils. References for the identification of soil fungi should be used to identify fungi isolated from roots. For example, see Tousson et al. (1970) and Singleton et al. (1992).

### **8.5.8 Isolation of Fungi Associated with Roots (Ectomycorrhizae)**

Ectomycorrhizae can be detected by their intimate association on host roots as well as when they produce fruiting bodies above ground. In identifying ectomycorrhizal fungi from roots, accurate host plant identification is essential, thus roots must be traced from plant bases. Both woody and fine roots will be sampled. Final identification based solely on the root-fungus structure is beyond the scope of this plan. At the current state of knowledge, such identification requires molecular methods.

#### **Requirements**

- a If option 8.5.8b is implemented, then the following protocol shall apply:
  - i Collection shall occur only once from each host plant sampled.
  - ii In cloud forest sampling of fine roots shall take place in the relatively dry season.

Note: The seasons can influence the ability to detect and isolate ectomycorrhizal fungi from fine roots.

- iii In all habitats, sampling of aerial roots shall occur in the wet season.
- iv In all cases, sampled roots shall be traced back to the base of the plant from which they issue in order to provide accurate host determination.
- v Fine root samples shall be gently washed in a 0.1% solution of sodium pyrophosphate and examined under a dissecting microscope for the presence of ectomycorrhizal root tips.
- vi Root tips showing the presence of ectomycorrhizae shall have their morphology described and shall be photographed on a neutral gray background.
- vii Voucher specimens of all collected ectomycorrhizae shall be preserved by freeze-drying.

Note: Identification of ectomycorrhizae from fungal sheaths on root tips requires the use of molecular methods. Many ectomycorrhizal fungi cannot be cultured from tissue of fruiting bodies, and spores of these fungi often will not germinate in culture.

### **Options**

- b To form a stock of material from which to make future identification of ectomycorrhizae from fine roots, i.e., those less than 2 mm diam, 10-20 cm lengths may be cut from roots of the same host species as those sampled for wood endophytes (8.5.6a).

## **8.5.9 Isolation of Fungi Associated with Roots (Endomycorrhizae)**

All plant communities include an assemblage of arbuscular endomycorrhizal fungi which are symbiotic with the majority of vascular plant species. Endomycorrhizal fungi are identified by the spores they produce in the rhizosphere. Rhizosphere samples can be collected by parataxonomists with a minimum of training.

### **Requirements**

- a Rhizosphere samples shall be collected at both ends of each sampling unit established for sampling terrestrial fungi in each habitat (chapter 5).
- b When recommendation 8.5.9y is implemented, additional sampling locations shall be established and used as follows:
  - i A permanent marker post shall be driven into the ground and labeled as required in 5.1.
  - ii This permanent marker post (8.5.9b(i)) shall have its GPS coordinates recorded as required in 5.1.
  - iii The marker (8.5.9b(i)) shall determine one end of a 245 m line the compass bearing of which shall be written indelibly on a label attached to the post in some permanent way as in 5.1.
  - iv Two samples shall be taken, one at each end of the 245 m line (8.5.9b(iii)).
- c Each sampling unit or additional sampling location (8.5.9y) shall be sampled only once as long as sufficient spores for identification are successfully extracted; and this sampling shall be near the end of the active growing season of herbaceous plants in the sampling unit or additional sampling location.

Note: The optimum sampling time is near the end of the active growing season of herbaceous plants in each plot. However, seasonal sampling may be needed to detect sporulation events and collect enough spores to make an identification.

- d Collection of rhizosphere samples shall be restricted to roots of, and soil around, herbaceous plants.

Note: This is to ensure that a maximum number of fungal propagules are obtained.

- e At sites where no herbaceous understory is present or where it is too sparse, five to ten bait plants shall be established in plots.
- f Half of all established bait plants (8.5.9e) shall be collected after 30 days growth and transplanted to pots in a greenhouse.
- g The other half of the established bait plants (8.5.9e, 8.5.9f) shall be left to grow to maturity, and spores shall be extracted directly from field rhizosphere samples.
- h Rhizosphere samples shall consist of 200 cm<sup>3</sup> living roots and attached soil from 3-4 selected herbaceous plants with abundant roots.
- i Each soil-root mix shall be placed in a plastic bag that then shall be closed and transported to a greenhouse for establishment of pot cultures with which to trap, and increase sporulation by, the fungi present in the sample.
- j To establish pot cultures, roots of each sample shall be chopped into fragments about 3 mm in maximum dimension with a hatchet or large scissors that has first been dipped in 95% alcohol and air dried.

Note: The root fragments are not to be sterilized or touched by any chemical treatment so that both internal and external hyphae remain viable and infectious.

- k The chopped root fragments (8.5.9j) are remixed with the soil from which they came to serve as the infectious inoculum in pot cultures.
- l The root-soil mixture (8.5.9k) shall be combined 1:1 (v/v) with autoclaved coarse sand consisting of 1 - 2 mm diameter or larger particles and placed in plastic pots 15 - 20 cm diameter that shall have been presterilized by immersion in 10% bleach for at least 30 min.
- m Pots containing the mix of autoclaved sand and root-soil mixture (8.5.9l) shall be seeded with Sudan grass or a more adapted tropical C<sub>4</sub> grass species.

Note: Bahia grass has been used successfully in Florida where greenhouse temperatures are routinely above 30 deg. C.

- n After seeding (8.5.9m), pot cultures shall be maintained in a greenhouse for a minimum of 4 months.
- o Fertilization of the pots (8.5.9m) shall be minimized. Fertilizer shall be applied only when plants show signs of phosphorus and nitrogen deficiencies appearing as reddening or yellowing of leaf sheaths, respectively.
- p To extract spores of arbuscular mycorrhizal fungi, the following steps shall be followed:
  - i A small portion of the potting medium (8.5.9l), usually 50-100 cm<sup>3</sup>, shall be removed using a sterile knife or small spade and placed in a beaker with 250 - 500 ml water.
  - ii After swirling to break up soil aggregates, the beaker contents shall be washed through two nested sieves—the top one with 500 µm openings and the bottom one with 38 or 45 µm openings.
  - iii The material retained by the lower sieve shall be placed in 50 ml centrifuge tubes containing a sucrose gradient with concentrations of 20% and 60% and shall be centrifuged at 900G for 3 min. and then decanted into a sieve with 38 or 45 µm openings and washed for one to two min. with sterile water to remove the sucrose
  - iv The filtrate shall be transferred to a glass Petri dish and refrigerated
  - v Spores shall be collected within three to four days after extraction.

Note: For more details concerning sucrose extraction see Daniels and Skipper (1982).

- q Spores of each morphotype shall be collected in water using a glass pipette modified to reduce the size of the tip opening and placed in glass vials with 0.5% sodium azide and stored at 4 deg. C.
- r From spores of each morphotype, a voucher specimen for identification and future reference shall be prepared as follows:
  - i A minimum of 20 spores shall be placed on a glass slide in permanent mountant per the double cover-glass method (7.4.1), but the slide shall not be sealed.
  - ii The spores shall be broken to reveal diagnostic characters by putting pressure on the cover slip.

- iii The slide shall be sealed following the double cover-glass method (7.4.1) and stored at room temperature.
- iv Digitized color images shall be prepared of representative specimens for each species found.
- s After the voucher slide is prepared (8.5.9r), pots shall be left to dry undisturbed in the greenhouse until plants completely wilt.
- t Once the potting medium is dry (8.5.9s), it is divided into four sections by making two vertical cuts through it at right angles to each other and stored in sealed plastic bags at 22 - 24 deg. C.
- u To confirm species identity, a 250 cm<sup>3</sup> sample of each trap pot culture established at INBio shall be mailed to INVAM (International Culture Collection of Arbuscular and VA Mycorrhizal Fungi, West Virginia University).

Note: Arbuscular endomycorrhizal fungi in all genera except *Gigaspora* can survive in this medium for two to three years. *Gigaspora* species will survive only six months and then must be recultured.

Note: The initial development of pot cultures of ACG specimens will take place in West Virginia. Experience through extensive training is needed to establish and maintain cultures, to isolate and identify morphospecies, and to set up voucher specimens. An individual with a biology/microbiology background can obtain the necessary expertise by 2-3 months of training at INVAM. After their development during the training period, the isolates from the pilot program will be returned to INBio and will become part of a curated culture collection (7.5).

- v If recommendation 8.5.9x is implemented then the preliminary sampling shall be carried out as follows:
  - i Eight samples shall be randomly collected from a burned area, a primary forest, and a secondary forest in the dry lowlands and up to 400 m.
  - ii Eight additional samples shall be collected from wet, rain, and cloud forest regions of the ACG. These samples shall be transported to INVAM, where they shall be processed as described above.

Note: Pilot program studies (3.2) are recommended to ascertain the breadth of arbuscular fungal diversity in selected habitats, and to determine if other trapping procedures need to be implemented (e.g., successive reseeded of trap pot cultures to stimulate sporulation).

Note: Spores of individual species separated from trap pot cultures extractions will be inoculated directly onto roots of 10 - 12 day-old sorghum or Bahia

grass seedlings that are transplanted into smaller pots to produce mono-specific pot cultures.

- w The ATBI's Head Mycologist, his/her direct staff (3.3.1), and relevant taxonomic experts from INVAM and the INVAM-trained coordinating staff member in Costa Rica (12.8.1) shall be responsible for deciding when resampling is necessary.

### **Recommendations**

- x A preliminary sampling for endomycorrhizae should be conducted at the beginning of the two-year pilot program.
- y In any region in which sampling units are not established and where noticeable change in plant community composition is noted, sampling locations for arbuscular endomycorrhizal fungi should be established.

## **8.5.10 Isolation of Fungi Associated with Roots of Ericaceous Plants**

Roots of ericaceous plants harbor a special kind of mycorrhizae that should be isolated using different techniques from those described above.

### **Requirements**

- a After surface sterilizing a sample of root, the sample shall be placed in a blender to macerate until cells have separated.
- b Following maceration (8.5.10a) the protocols of Leake and Read (1994) shall be followed.

## **8.5.11 Non-Culturable Endophytic Fungi in Living Plant Tissue.**

Associated with living plant tissues are a suite of species of non-culturable, endophytic fungi. These fungi may be visible as hyphae or may only be detected using molecular methods unless they can be recognized from direct observation of dead material in subsequent saprobic growth phases. Current knowledge is not sufficient to identify fungi in this way, but studies of molecular variation may provide an indication of the proportion of species that are not detected by traditional means (Appendix C).



## **Sampling Protocols—Fungi Associated with Dead or Recently Cut Wood and Woody Substrates**

Fungi are the major decomposers of woody substrates in both temperate and tropical ecosystems. The species involved belong to all major groups of fungi and range in size from large, bracket fungi and mushrooms to specialized yeasts. In addition many fungal species are associated with woody substrates even though they are not decomposing the wood. These fungi occur, for example, as parasites or saprophytes on the wood-decaying fungi, on insect frass or in insect galleries, and as part of microbial mats that develop within moist pockets in decaying wood. Of the 50,000 fungal species estimated to occur in the ACG, those associated with woody substrates probably number about 12,000 species. Some of the fungi associated indirectly with wood will be obtained when sampling non-woody substrates while others will be located only by the long-term monitoring of decaying wood that is part of the sampling strategy for wood and woody substrates. An inventory of wood-associated fungi must include sampling the diversity of woody plant species in all stages of decay in all vegetation zones. Large, decay-resistant trees may take over a hundred years to completely decompose. During the process of decomposition, the structure is transformed following tree death through gradual decay on the forest floor, eventually becoming integrated with the soil. At present little data exist on rates of wood decay in tropical regions.

### **9.1 Sampling Protocols for Fungi Associated with Dead or Recently Cut Wood and Woody Substrates**

#### **9.1.1 Sampling Protocols for Fungi Associated with Dead or Recently Cut Wood and Woody Substrates—Common Protocols**

##### **Requirements**

Note: Macromycetes must be collected and processed in accord with 6.1 and 6.2. Myxomycetes must be collected and processed in accord with 6.1, 6.4, 6.5. Lichenized fungi must be collected and processed in accord with 6.1.

- a When sampling fungi associated with wood using the various approaches described below, the decay state (class) of the wood from which the fungi are sampled or isolated shall be recorded in the specimen description according to the following classification system:

- i dying to partly alive (DPA)—recently killed, with bark; rot or substrate softening penetrates a maximum of 3 cm into wood

Note: To evaluate depth of rot penetration, probe with a knife.

- ii in moderate state of decay (MSD)—partially decorticated; wood softened by rot to depth greater than 3 cm; hard core of wood still present
  - iii in advanced state of decay (ASD)—easily cut; wood completely softened by rot; possible to tear substrate apart with knife.
- b Wood from which a specimen is collected shall be collected with the fungus to allow identification of the substrate.

Note: Wood identification may be carried out on-site with assistance from the Plant TWIG or by later comparison in the laboratory with known wood samples such as those maintained at the Forest Products Laboratory, University of Costa Rica.

- c Sampling for lichenized fungi shall follow the methods given in 8.4.3f through 8.4.3m and 8.5.3.

### **Recommendations**

- d Among the terms used to define position of a woody substrate relative to the ground should be the following: standing, broken, uprooted, suspended (separated into two subcategories—less than 1 m above ground and more than 1 m above ground), tree contact, partial ground contact, and on ground.
- e Dimensions of woody substrates of a collected specimen should be recorded in the specimen description as well as the position of the substrate relative to the ground.
- f If the woody substrate of a collected specimen is *not* attacked by insects, this should be recorded in the specimen description.
- g The moisture content of the wood from which a specimen is collected should be measured by a Shigometer (Shigo and Shigo, 1974) and recorded in the specimen description.

## **9.1.2 Monitoring Actively Felled Trees—Common Protocols**

Collecting fungi on actively felled trees has two major advantages. First, the species identity of the host substrate can be determined when the tree is still alive. Second, the anticipated rapid species turnover during the first phase of decay can be followed carefully. Collecting on wood in moderate and advanced

states of decay will be conducted in plots for naturally fallen trees (9.1.5) and by opportunistic sampling (9.1.7).

### **Requirements**

- a Living trees shall be felled and monitored for long-term succession of fungi.
- b At least two tree species, preferably of different genera, of each family with woody plants shall be selected for sampling, with more than two taxa selected from the largest families; and one to three individuals of each selected species shall be felled and studied.

Note: This is equivalent to two individuals each of approximately 200 representative species—approximately 400 trees total for all vegetation zones.

- c Of each species to be felled, one specimen shall be cut before a rainy season; and one specimen shall be cut after a rainy season.
- d Each felled tree shall be given a unique identifier.
- e From each cut tree, samples of healthy wood shall be selected, catalogued, and filed as vouchers.
- f During the initial six months after a tree is felled especially in the wet and cloud forests, the substrates
  - i shall be visited every week and systematically examined for the presence of fleshy fruiting bodies
  - ii shall be visited at least every two weeks and examined for the presence of polypores and other Aphylophorales.

### **Recommendations**

- g When selecting tree species to be cut, the maximum spectrum of specific wood characteristics (especially durability) should be covered.
- h Once the initial six month sampling period is over, sampling should occur at least four times per year for the next 18 to 30 months; in dry forest, sampling should occur twice each during the wet and dry seasons.
- i Once the initial six month sampling period is over, sampling should occur more frequently in wet and cloud forests than in dry forest (9.1.2h) because of the rapidity with which fleshy fungi decompose in moist environments.

### 9.1.3 Sampling Actively Felled Trees by Indirect Observation

Note: All cultures are to be preserved in the INBio culture collection (7.3.2).

#### Requirements

- a In the manner described for sampling woody substrates for endophytes (8.5.6), samples for culturing shall be obtained from the decaying woody substrates.
- b Starting when the trees are felled, such samples shall be obtained once every two weeks for the first six months and, thereafter, shall be obtained every two to six months depending on the results:
  - i Bark samples shall measure about  $20 \times 5$  cm.
  - ii Using an increment borer, wood core sections shall be taken from the bottom, midsection, and top of each felled tree.

Note: Because rapid changes may occur during the initial six month period after the tree is felled, more frequent sampling is required during that period.

- c Wood core sections shall be placed in sterilized containers, and transported to the laboratory.
- d Isolations shall be made from the core sections (9.1.3b(ii)) on standard media and axenic cultures obtained.
- e Procedures and media for isolating from wood cores shall be based on intensive isolation methods using pulverized samples (7.3).

### 9.1.4 Sampling Actively Felled Trees by Direct Observation in Moist Chambers

#### Requirements

- a Portions of each bark and core sample (9.1.3b, 9.1.3c) shall be incubated in moist chambers for as long as new *Myxomycetes* or fungal species sporulate on the substrates.
- b For each such substrate, notes shall be taken on all *Myxomycetes* or fungal species in situ.
- c Any *Myxomycetes* sporulating shall be segregated still attached to substrate, allowed to mature, and collected in accord with 8.4.4.
- d From each fungal species, the reproductive bodies shall be collected and cultures of them shall be obtained.
- e Axenic cultures of collected fungi shall be maintained on standard media as follows:
  - i Wood decaying Basidiomycetes shall be grown and stored on Malt Extract Agar (7.2b(ix)).

- ii Ascomycetes and conidial fungi shall be grown and stored on Cornmeal Agar (7.2b(iii)), Cornmeal Dextrose Agar (7.2b(iv)), or Dilute Malt Extract Agar (7.2b(vii)).

### **9.1.5 Monitoring and Sampling Naturally Fallen Trees— Common Protocols**

#### **Requirements**

- a Decaying trees that are standing dead or have already fallen prior to the start of the Fungus ATBI shall be mapped and marked for a defined area of one hectare within each vegetation zone.
- b The area to be mapped shall be adjacent to the area with the transects established according to the protocols of chapter 5.
- c A data base of these decaying and previously fallen trees with details of their characteristics shall be established as part of the Plant TWIG.
- d These trees shall be sampled in the manner described above (9.1.2 through 9.1.4) for purposely fallen trees.

### **9.1.6 Sampling Large Woody Substrates in Sampling Unit Plots— Common Protocols**

#### **Requirements**

- a Wood-associated fungi shall be sampled from selected plots in sampling units (chapter 5) in each vegetation zone.
- b Two of the selected plots within each vegetation zone shall be sampled for wood-associated fungi once a month during the rainy season and every three months (i.e., twice) during the dry season.
- c A subset of branches < 5 cm diameter collected during sampling of terrestrial and soil substrates (10.1.1) shall be segregated for isolation and culturing following the same methods used for larger diameter branches in 9.1.6e and 9.1.6f.
- d Sampling a plot for wood-associated fungi shall consist of
  - i examining all portions of woody substrates > 5 cm diameter.
  - ii collecting all fungal fruiting bodies and sporulating colonies on such substrates in good condition
  - iii collecting 50 cm segments of ten different branches over 5 cm diameter.
- e *Isolation from Woody Substrates in Terrestrial Plots.* 10 cm long portions of each of the ten branches from each plot sampled for woody substrates (9.1.6d) shall be sectioned, surface sterilized (7.1), and

plated on general and selective media using the procedures described for the isolation of fungi from living woody substrates (8.5.6).

- f *Moist Chamber Incubation of Woody Substrates.* The remaining 40 cm long portions of the ten branches from each sampled plot (9.1.6d, 9.1.6e) shall be sectioned further if necessary and placed in moist chambers and monitored periodically for sporulating fungi following the methods described in 10.1.4f through 10.1.4l.

### 9.1.7 Opportunistic Sampling from Large Woody Substrates

Considerable numbers of fungal species will be obtained by opportunistic sampling (i.e., wandering through the forest looking for sporocarps).

#### Requirements

- a Parataxonomists shall collect for 3-4 hours each day during site visits, ensuring as much as possible that the sampling path does not cover the same ground more than once each visit.
- b Collecting shall focus primarily on macrofungi visible without a hand lens, particularly those on woody substrates in later stages of decay.

Note: It is often necessary to examine the under-surface of woody substrates for sporulating fungi.

Note: Woody substrates in earlier stages of decay will be sampled by methods of sections 9.1.2 through 9.1.6. Identification aids for the woody tree species of the ACG will be needed. It is particularly difficult to identify species of well-decayed woody substrates.

- c In addition to recording data about the specimen itself and the substrate, the exact location of each collection shall be recorded using GPS.
- d After collecting, the remaining 4-5 hours of the day shall be devoted to making spore prints, tissue cultures, and preparation of collected samples for deposition in herbaria.
- e Each of the eight vegetation zones (1.5) shall be visited six times every year.
- f The time invested in each vegetation zone shall be proportional to the species richness of woody plants in that zone.

## **Sampling Protocols—Fungi Associated with Terrestrial, Soil, Rock, and Aquatic Substrates**

Fungi associated with terrestrial, non-living plant, and soil substrates can be sampled by direct observation and by culturing from these substrates. The major groups of fungi inhabiting litter and soil include Agaricales, Aphyllophorales, Gasteromycetes, stromatic Ascomycetes, Pyrenomycetes, Discomycetes, Deuteromycetes, and Myxomycetes. Terrestrial, non-living plant and soil substrates may harbor the vast majority of the diversity of fungi that exist. In addition, there is considerable overlap between the fungi occurring on these substrates and those on living plant and woody substrates.

For macrofungi, sites are sampled periodically during the rainy season that lasts 5-7 months per year in dry forests and throughout the year in humid areas. Because of the annual variation in fruiting of individual species (some do not fruit every year or even every five years), a small number of species that actually occur in the sampling units probably will not be collected during the 5 year, full scale Fungus ATBI program. However, by intensively sampling all vegetation types throughout the fungal fruiting season for multiple years, most species will be encountered. Additional sampling is planned to include habitats or microhabitats with unique taxa such as epiphytic humus mats or habitats expected to be unusually species-rich.

All major taxonomic groups of fungi are encountered within litter and soils. Direct observation of many fungi in soil and well-decomposed plant litter is possible, but usually only indistinct and featureless mycelia are visible. Vegetative states of the fungal life cycle generally can only be reliably identified using species-specific probing techniques such as immunofluorescent labeling or indirectly via nucleic acid probes. Therefore, taxonomic surveys of soil fungi rely heavily on isolation techniques in which fungal propagules such as spores, sclerotia, and mycelial fragments, are extracted from soil and induced to grow and develop identifiable reproductive structures on culture media. Although this indirect cultural approach has technical limitations and interpretation of results can be difficult, it is the preferred method for evaluating the taxonomic composition of the soil mycota.

Density of fungal propagules and fungal biomass, and therefore perception of species composition of soil fungi, fluctuate seasonally, especially in relation to rainfall and soil moisture. In addition, changes will occur due to plant and

fungus succession. Thus, the Fungus ATBI will capture much, but not all, of the diversity of soil fungi.

An enormous body of information already exists for soil fungi, although this knowledge is incomplete, especially for tropical biomes. Thus, a reasonably complete inventory of the saprobic soil mycota of the ACG can be achieved within five years.

In addition to dealing with fungi of terrestrial substrates, this section describes protocols for sampling fresh water in streams and splash cups in which the distinctive aquatic and aero-aquatic fungi occur. In addition, rocks serve as substrates bearing a diversity of lichenized fungi that can be sampled by direct observation.

## **10.1 Sampling Protocols For Fungi Associated with Terrestrial, Soil, and Rock Substrates**

The sampling protocol for terrestrial substrates is designed first and foremost to ensure the sampling of all fungi associated with these substrates. Over time, the efficiency of terrestrial sampling may be improved as results are analyzed and modified to reduce duplication of effort. The overall sampling strategy is based on a series of sampling units representative of the major vegetation zones (1.5) plus auxiliary plots deliberately selected to target specialized soil, parent materials or stands of vegetation (chapter 5). For the two-year pilot program (3.2), 14 or 15 sites representative of the vegetation types will be established and sampled (5.1g), however, for the five-year, full scale Fungus ATBI, 52 sites will be established with at least two sampling units within each vegetation type (5.2a). The number of sites in each vegetation type was based on the anticipated diversity of fungi to be encountered and the relative area of each habitat. The entire ACG sampling is stratified by vegetation type; more area is sampled in particularly extensive habitats, e.g., dry forest, and those predicted to yield highest diversity, e.g., rain forest.

### **10.1.1 Sampling Terrestrial, Soil, and Rock Associated Fungi—Common Protocols**

#### **Requirements**

Note: Macromycetes must be collected and processed in accord with 6.1 and 6.2. Myxomycetes must be collected and processed in accord with 6.1, 6.4 and 6.5. Lichenized fungi must be collected and processed in accord with 6.1. Fungi on wood > 5 cm in diameter occurring in the plots, are covered by the protocols concerning sampling large wood or woody substrates found in terrestrial plots (9.1.6).

- a In the first rainy season, at the beginning of the pilot program, before the sampling of the plots described in 5.1 begins, there shall be a two

week sampling effort for macromycetes, Myxomycetes, and lichens only. During these two weeks, ten 1m<sup>2</sup> plots shall be sampled in dry forest habitats and ten 1m<sup>2</sup> plots shall be sampled in low elevation rain forest habitats (Table 1).

- b During this period, opportunistic collecting of perennial sporocarps, Myxomycetes, and lichens outside of the 20 plots shall be encouraged.

Note: The purpose of this preliminary sampling (10.1.1a) is to quickly assess the overlap (6.6.1) in species between the two habitats and to decide, based on this overlap, if an additional plot is required in either habitat or both. The purpose of opportunistic sampling of specific organisms is to obtain rapidly more nearly complete lists for three groups of fungi that are often and easily seen, for which much data are already available, and for which the number of novel organisms is not expected to be as high as with other groups.

Note: Information from the twenty 1m<sup>2</sup> plots sample (10.1.1a) will also be useful in selecting rich areas on which to establish sampling units.

- c After assessing the collections from the initial trial period (10.1.1a, 10.1.1b), if the overlap of species present in the habitats (6.6.1, 10.1.1a) is less than 40%, then three sampling units shall be established in the “rain forest - low” habitat during the pilot program (3.2, 5.1, Table 1); otherwise, two sampling units shall be established in this habitat during the pilot program.

Note: The number of sampling units per habitat is provided in Table 1.

- d After the initial trial period (10.1.1a through 10.1.1c), each site shall be sampled every two weeks beginning five days after the onset of a rainy season and continuing throughout that season.
- e The sampling methods of 6.1 through 6.4 shall apply.
- f In order to detect the maximum number of species, collectors shall give special attention to collecting inconspicuous taxa.
- g All fungal sporocarps > 1 cm in some dimension shall be removed from every plot except for limitations imposed by stop rules in effect.

Note: An area of 400 m<sup>2</sup> per sampling unit is sampled for sporocarps > 1 cm in some dimension. Collecting all sporocarps from these units is essential because many species cannot be easily distinguished in the field.

- h All sporocarps of smaller fungi (i.e., < 1 cm in all dimensions and visible on hands and knees without magnification), shall be removed from every fifth plot in a sampling unit beginning with plot number 5 (Fig. 3) or as directed by stop rules that are in effect.

Note: An area of 80 m<sup>2</sup> per sampling unit will be sampled for smaller sporocarps. Collecting all sporocarps from these areas is essential because many species cannot be easily distinguished in the field.

Note: Any given stop rule will apply to the collecting of a specific taxon or specific groups of taxa within a specific sampling unit or specific groups of sampling units.

- i In so far as possible, collections shall be sorted by taxon as each plot is sampled.
- j When material is collected opportunistically (per 10.1.1i), such collections should be labeled with latitude and longitude (GPS) and collecting locality with an indication that the specimens did not come from a plot.
- k The litter of selected host species such as palms, members of the Musaceae, and tree ferns shall be targeted and sampled even though such litter may not occur in the plots of a specific vegetation zone sampling unit.

Note: Some host species are known to be unusually rich in microfungi, but these fungi may occur only rarely in the Fungus ATBI plots. The selection of a complete list of such host species is beyond the scope of this Plan.

- l At least in the odd numbered years of the ACG Fungus ATBI, at least once in the rainy season and at least once in the dry season, one soil sample (10.1.6) and one leaf litter sample (10.1.4) shall be taken from each sampling unit from an area not otherwise used for sampling for intensive isolation of fungi by sample pulverization methods (10.1.7).
- m At least once each during the Fungus ATBI, more specialized types of soils (e.g., river silts, pond and puddle silts, epiphytic humus and soils associated with epiphytic plants and large canopy branches) shall be collected and examined.

Note: The development of the list of such specialized soils is the responsibility of the Head Mycologist, his/her direct staff, and the Expert Taxonomists Group of the Fungus TWIG (3.3.1).

- n After one sampling season, the sampling techniques shall be evaluated to determine species-effort curves.

Note: A species-effort curve will suggest when most species have been detected and the sampling can be modified or terminated.

- o When recommendation 10.1.1r is carried out, it shall be constrained by any stop rules then in effect.

- p After one sampling season, for each sampling unit, sampling data shall be used to determine percent overlap (6.6.1) between:
  - i lists of taxa present in sequential pairs of plots along each transect
  - ii lists of taxa present along the two transects within the sampling unit (based on the same data)
  - iii list of taxa present in the sampling unit at sequential time intervals.
- q When evaluating the sampling techniques (10.1.1n, 10.1.1p), either of the following shall indicate that less intensive sampling is sufficient during subsequent years and a stop rule (3.3.2a, 3.3.2b) shall be developed and invoked as appropriate:
  - i a species-effort curve showing a distinct shoulder—a marked decrease in slope, a leveling
  - ii greater than 60% overlap (6.6.1) in one of the comparison categories listed in 10.1.1p.

Note: This level of overlap (10.1.1q(ii)) indicates that less intensive sampling is sufficient *on the relevant scale*. Consider a number of examples: Greater than 60% overlap between a pair of samplings sequential in time suggests that less frequent sampling may be appropriate. If greater than 60% overlap between adjacent plots on the same date is noted, then sampling of every other plot may be sufficient. If less than 40% overlap in species lists within a plot for sequential sampling dates is observed, then sampling should occur more frequently. If more than 60% overlap in large mushrooms is noted between transects in the same sampling unit, then one could consider dropping one of the transects.

### **Recommendations**

- r Opportunistic collecting outside of actual plots should be carried out.

### **Options**

- s When evaluating the sampling techniques (10.1.1n, 10.1.1p), more intensive sampling may be instituted on the relevant scale (see above note under 10.1.1p) if less than 40% overlap (6.6.1) in one of the comparison categories listed in 10.1.1p.

## **10.1.2 Sampling Plots for Macrofungi**

### **Requirements**

- a The standard protocols for collection of macrofungi (6.1, 6.2) shall be augmented by specialized collecting, trapping, or culturing techniques using specialized media for Basidiomycetes such as Benomyl

Dichloran Streptomycin (7.2b(i)), to detect taxa that might otherwise be missed (11.3.2g(iv)).

**10.1.3 Sampling Plots for Myxomycetes**

**Requirements**

- a The standard protocols for collection of Myxomycetes (6.4) shall be augmented by direct observation via the appearance of these organisms during monitoring of litter in moist chambers (10.1.4).

**10.1.4 Sampling Plots for Plant Litter and Isolation of Microfungi in Moist Chambers**

**Requirements**

- a “Fresh litter” shall be construed to include identifiable plant parts such as dead leaves, fruits, seeds, and wood < 5 cm diam.

Table 2. Choice of reference plot based on number of visits to a sampling unit

| Sampling Visit Number*   | Reference Plot Numbers |
|--|------------------------|
| 1, 11, 21, 31, 41, 51  | 1, 11, 90, 100         |
| 2, 12, 22, 32, 42, 52  | 2, 12, 89, 99          |
| 3, 13, 23, 33, 43, 53  | 3, 13, 88, 98          |
| 4, 14, 24, 34, 44, 54  | 4, 14, 87, 97          |
| 5, 15, 25, 35, 45, 55  | 5, 15, 86, 96          |
| 6, 16, 26, 36, 46, 56  | 6, 16, 85, 95          |
| 7, 17, 27, 37, 47, 57  | 7, 17, 84, 94          |
| 8, 18, 28, 38, 48, 58  | 8, 18, 83, 93          |
| 9, 19, 29, 39, 49, 59  | 9, 19, 82, 92          |
| 10, 20, 30, 40, 50, 60   | 10, 20, 81, 91         |
| * After 60 site visits, begin again at the top of the table with reference plots for visit 61 being the same as for visit 1. |                        |

- b On each transect (Fig. 3), fresh litter shall be removed from 4m<sup>2</sup> on each site visit by the procedure defined as follows:

Note: Plots on the transects are numbered as described in 5.1 and depicted in Fig. 3.

- i On each visit to the sampling unit, four reference plots shall be selected to define litter collecting areas by the use of Table 2.

Note: These plots are all at one end of the transects to reduce the difficulty of carrying the bags of litter from the sampling unit to the transport. On the other hand, the distance between the reference plots is set at 50 m to increase the number of taxa that may be recovered by the process.

- ii When and only when a plot is a reference plot (10.1.4b(i)), all fresh litter shall be collected from within a single 1 m<sup>2</sup> frame positioned based on the number of times the plot has been visited for sampling using the combination of Table 2, Table 3, and Fig. 4; “left” and “right” shall be determined by standing on the transect including the given plot facing in the direction of increasing plot number.
- c Collected plant litter shall be placed in bags for transport to Santa Rosa to be examined for microfungi. Plant litter components shall be identified, if possible, to plant species and plant part.
- d No reference plot shall have a sampling frame placed in a given offset position more than once per year.

Table 3. Direction of offset from center of reference plot for placement of 1 square meter frame within which litter is to be collected

| Sampling Visit Number*   | Offset Position** |
|--|-------------------|
| 1 - 10   | right             |
| 11 - 20  | right forward     |
| 21 - 30  | left forward      |
| 31 - 40  | left              |
| 41 - 50  | left back         |
| 51 - 60  | right back        |
| * After 60 site visits, begin again at the top of the table with reference plots for visit 61 being the same as for visit 1. |                   |
| ** Offset positions are defined in Fig. 4.   |                   |

- e At Santa Rosa, plant litter from the plots shall be observed and sorted, piece by piece, with the aid of a dissecting microscope (80× or greater); detected microfungi shall be dissected from the substrate; and permanent slides shall be prepared (7.4.1).
- f After examination, plant litter shall be placed in a moist chamber at ambient temperature and monitored for two weeks; during this two week period, microfungi shall be collected as they appear. Moist

chambers shall be made from large plastic boxes lined with moist vermiculite.

- g Paradichlorobenzene shall be added to chambers to kill mites and insects.

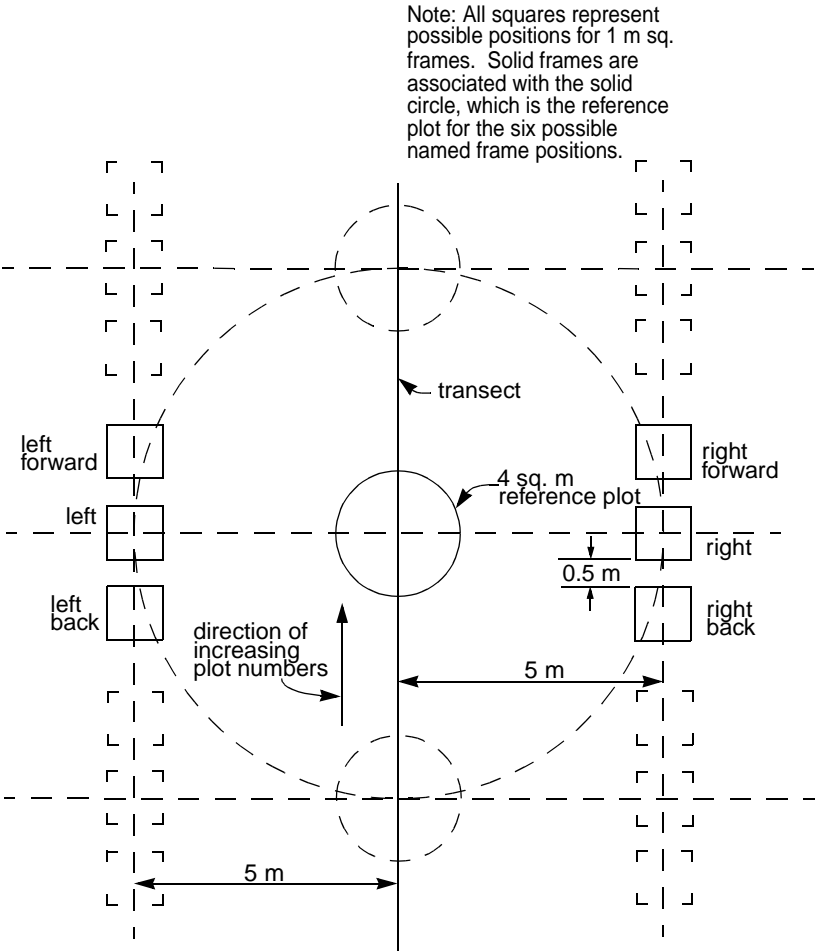


Fig. 4. Schematic showing positions of 1 square meter frame with relation to center of a reference plot

- h Myxomycetes forming sporocarps in the moist chambers shall be collected following the protocols of 6.4.

- i As microfungi are observed or develop in a given moist chamber, taxa shall be cultured from spores or living tissue when such taxa have not previously appeared in the current litter sample unless a relevant stop rule is in effect (3.3.2a, 3.3.2b).
- j Because it is impossible to culture from all sporocarps, emphasis shall be placed on obtaining representative cultures from each newly observed taxon; and stop rules shall be developed (3.3.2a, 3.3.2b) to limit the repeated culturing of fungi common to a sampling unit.

Note: Culturing is often required to obtain complete morphological information for identification, to study the vegetative state and colony morphology, and to determine alternate sporulating states not evident at the time of initial observation. Culturing from identified sporocarps may facilitate identification of sterile cultures derived from litter, wood and other substrates.

- k After culturing as required (10.1.4h, 10.1.4j), microfungi with accompanying substrates shall be dried and placed in packets with a tentative identification.
- l Stop rules shall be developed to prevent preparation of an excessive number of specimens and permanent slides of any single taxon.

### **10.1.5 Direct Observation of Lichenized Fungi on Rocks**

- a Lichenized fungi on soil and rocks including submerged rocks shall be sampled at selected sites.

Note: Protocols for collection of lichenized fungi are covered by the general requirements in 6.1.

- b The selection of such sites shall take into account such factors as the presence of different types of soil or rocks (with the aid of geological maps), the quantity and size of the rock present at the potential sites, presence of microclimate variations such as exposure, shade, humidity, surrounding vegetation, and the conspicuous presence of lichens.

Note: The sites for sampling other fungi will not provide sufficient opportunities to sample lichens. Additional sites will be necessary, in rocky areas on hill tops, steep slopes, ravines, and along streams. Since soil lichens are usually confined to open sites, they can often be observed adequately on the same sites as rock lichens.

- c In the selected sites and for each available type of microclimate within such sites, rock or soil surfaces of from several dm<sup>2</sup> to about 1 m<sup>2</sup> shall be inspected with the aid of a hand lens, for lichenized fungi.
- d Special attention shall be paid to sites with temporary or more permanent water run-off.

- e Crustose rock lichens shall be collected including pieces of attached rock, if necessary, with the aid of hammer and chisel.

**Options**

- f Since lichens are long-lived and permanently visible, each site may be sampled only once.

**10.1.6 Collecting Soil for Isolation of Fungi Using Pulverized Sample Methods**

**Requirements**

- a Each sample of soil taken in the field and intended for use in isolation of soil fungi (10.1.11) shall weigh 10-30 g.
- b Each site shall be the source of 8-20 such soil samples (10.1.6a) over the seven year course of the ACG Fungus ATBI.
- c Sampling shall be by use of a soil coring tube or other tool sterilized in 70% ethanol and dried just prior to collection.
- d Collections shall be made from a profile or mineral soil surface, carefully exposed by removal of overlying litter and humus, if present.
- e The samples (10.1.6a) shall be
  - i placed in labeled, sterile plastic bags or plastic vials
  - ii transported in an ice chest or cooler (without ice) to the culture facility at INBio
  - iii refrigerated at 4 deg. C
  - iv plated out within 4 days if at all possible using the protocols of 7.3.1
  - v discarded if stored more than 7 weeks without being plated out.

Note: Upon return to the culture facility at INBio, each composite soil sample is examined using complementary isolation techniques to recover the broadest taxonomic range of fungi (7.3.1, 10.1.7).

- f If soil samples are discarded without having been plated out, the following shall be done:
  - i This fact shall be recorded.
  - ii Fungus ATBI management shall re-assess protocols with a goal toward preventing further waste of effort and material.
  - iii Fungus ATBI management shall assess whether the soil samples need to be replaced by re-collecting at the relevant sampling units.

### **10.1.7 Isolation of Fungi from Soil or Pulverized Leaf Particles**

#### **Requirements**

- a In the case of litter samples, leaves shall be segregated from all soil, wood particles, and other detritus before pulverization.
- b Soil and pulverized leaves shall be handled separately, but by the same protocol—the method for intensive isolation from pulverized samples (7.3).

Note: Upon return to the culture facility at INBio, each composite soil sample is examined using complementary isolation techniques to recover the broadest taxonomic range of fungi (7.3.1, 10.1.7).

## **10.2 Fungi in Aquatic Habitats**

Fungi in aquatic habitats are often highly specialized. Some species complete their life cycles in water while others use that medium only for dispersal or reproduction. The number of fungal species associated with aquatic environments is estimated to be about 800-1,200 species in the ACG, thus probably comprising a relatively low percentage of all fungi. Fungi in aquatic habitats include aquatic and aero-aquatic Hyphomycetes, often with specialized and complex conidia that tend to accumulate on bubbles by surface tension, aquatic Ascomycetes, and species in the Zygomycetes, chytrids and Oomycetes. Chytrids and protostistan taxa have complex life cycles that often require special enrichment techniques to detect their presence and induce expression of motile and reproductive phases of their life cycle. For these reasons, the inventory for fungi in aquatic substrates requires assistance from experts in these specialized groups.

The basic pattern of sampling for aquatic fungi follows a continuing circuit of visits to water courses and standing water of the ACG, concentrating on dry forest sites during the rainy season. Also, collecting activities should be coordinated with other TWIGs collecting fishes, aquatic invertebrates, and algae to save transportation costs, to identify potential fungal substrates and to ensure adequate coverage of all types of aquatic habitats. This coordination is especially important to optimize specialist in-country collecting. For example, we envision that visiting algal and tetrad specialists would coordinate their visits with the respective parataxonomists and curators.

### **10.2.1 In-situ Detection of Aquatic and Aero-aquatic Hyphomycetes**

#### **Requirements**

- a The following aquatic habitats shall be sampled:

- i flowing permanent, seasonal, and temporary water bodies in all the 20 distinct ecosystems in the ACG such as temporary and seasonal small creeks on steep terrain, permanent creeks and streams, rain water flowing through the canopies of trees, and stem runoffs
- ii stagnant water bodies such as temporary or seasonal pools, bromeliad and other epiphyte tanks, water-filled tree hollows such as may be found in decapitated palms, *Agave* tanks, and permanent stagnant lakes or pools and swamps.
- b Sampling shall occur in all distinct habitats of the ACG listed in Table 1.
- c Samples shall be collected from aquatic sources 1-2 times yearly for a period of three years during the full-scale Fungus ATBI; and sampling effort shall be greatest during rainy seasons and especially at their outlets.
- d Foam developing at the surface shall be sampled from flowing water, from rainwater, from plant surfaces, or on the ground at the bases of trees after heavy rains.
- e If foam is not present on the aquatic source surface to be sampled, water samples from the source of approximately 0.1 to 1 liter shall be poured through a 200  $\mu$ m mesh sieve into a container with 0.2 to 20 ml formalin added.
- f After collecting, spores in a sample shall be left to settle for 24 hours.
- g After the 24 hour delay (10.2.1f) and after decanting off the supernatant, the remaining portion of the sample shall be concentrated in conical centrifuge vials.
- h Concentrated samples (10.2.1g) shall be fixed and mounted in semi-permanent slides for microscopic analysis.
- i Taxa shall be characterized by their distinctive spore shapes and permanent slides shall be prepared and sent for identification by expert taxonomists.

Note: Several international experts specialize in this group and a world monograph of the aero-aquatic Hyphomycetes is in preparation by J. Webster, UK, and E. Descals, Spain. An international expert cooperating with an in-country collector could complete the inventory of these fungi within a short period of time and produce a monograph and identification guide to these fungi in the ACG.

- j Specimens shall be retained as permanent microscopic slides.

## **10.2.2 Direct Observation of Aquatic and Aero-aquatic Ascomycetes**

### **Requirements**

- a Semi-submerged litter and small diameter wood samples shall be collected from all selected water bodies and taken to the laboratory for examination under the dissecting microscope.
- b If fungi are not sporulating, material shall be incubated in moist chambers and inspected at least once a week until the material is no longer in useful condition.
- c When sporulating material is noted in the moist chambers (10.2.2b),
  - i propagules shall be removed with a sterile needle and placed one per Petri plate on Cornmeal Agar (7.2b(iii)).
  - ii propagules or sporocarps of each taxon observed shall be mounted in a permanent slide using the double cover-glass method (7.4).
- d If culturing of aquatic Ascomycetes is required, the method used shall be that employed for terrestrial Ascomycetes (7.3.1f(iv), 9.1.4e(ii)).

Note: Culture of aquatic Ascomycetes is occasionally required for identification. Aquatic Ascomycetes can be grown in culture from single ascospores and may produce an asexual state.

## **10.2.3 Detection of Zygomycetes, Chytrids and Oomycetes in Aquatic Habitats**

### **Requirements**

- a Plant debris and algae shall be collected at or below the waterline of aquatic sources and placed in plastic bags in a cool chamber (e.g., ice chest without ice) until return to the laboratory.
- b Upon return to the laboratory, each of the samples shall be split into two parts.
- c Algae and plant debris shall be analyzed under the microscope.
- d To ensure adequate coverage of algal hosts, collecting shall be coordinated with the work of the Alga ATBI.
- e Permanent slides shall be made for subsequent identification from the first part (10.2.3b) of each sample.
- f The other part of each sample (10.2.3b) shall be subjected to baiting by incubating with pollen, hemp seeds, snake skin, or cleared grass.
- g Baits shall be observed under the microscope, and permanent slides (7.4) shall be made for subsequent identification by expert taxonomists.

- h If necessary and useful, taxa shall be cultured to determine life cycle characteristics.
- i Zygomycetes shall be cultured, if possible, by plating substrates on weak (one half to one fifth strength) Cornmeal agar.

Note: For those Zygomycetes that prove difficult to maintain in culture and for chytrids and Oomycetes, the best method for documenting collections is by permanent microscope slides.

#### **10.2.4 Isolation of Non-specialized Fungi in Aquatic Habitats**

##### **Requirements**

Note: All cultures are to be preserved in the INBio culture collection (7.3.2).

- a Periodically organic substrates from water courses shall be treated in the manner used to sample soil or litter (7.3, 10.1.4, 10.1.6, 10.1.7).

#### **10.2.5 Enrichment and Cultivation of Aquatic and Aero-aquatic Taxa**

##### **Requirements**

Note: All cultures are to be preserved in the INBio culture collection (7.3.2).

- a With regard to aquatic and aero-aquatic taxa, the first three years of the seven year ATBI project shall be directed towards obtaining a list of the taxa present.
- b In the last four years of the Fungus ATBI, the same sites shall be revisited to obtain isolates of important taxa or taxa for which cultures will further aid elucidation of life cycles.
- c In the last four years of the full-scale Fungus ATBI, plant material from water courses shall be brought back to the laboratory and placed in Petri dishes.
- d The Petri dishes containing the samples of plant material from water courses (10.2.5c) shall be
  - i flooded with sterile distilled water
  - ii gently agitated on a shaking platform at a moderate temperature (ca. 20 deg. C).
- e Plates (10.2.5c) shall be examined every few days for sporocarps or spores of aquatic and aero-aquatic fungi.
- f When they are found in the plates (10.2.5e), spores of aquatic and aero-aquatic fungi shall be transferred from the plates by hand or by micromanipulation to new agar to obtain pure cultures.

- g These purified cultures (10.2.5f) shall be accessioned to the culture collection (7.5).



## **Sampling Protocols—Fungi Associated with Animals and Animal Products**

### **11.1 Introduction**

A great variety of fungi are found in association with living or dead animals and animal products. The substrates included within this scope range from living, dying, and dead mammals and other vertebrates, insects, spiders, mites, and protozoa to their dung, nesting material, hair, scales, feathers, horn, and egg shells.

Associations with living animals can be divided into three groups based on the effect on the animal host, namely, commensal associations, in which the fungus derives benefit and the animal host is unaffected; mutualisms, in which both the fungus and host derive benefit from the association; and parasitism, in which the fungus derives benefit at the expense of the animal host.

The fungi associated with animals and animal products range from ubiquitous Zygomycetes, Ascomycetes, Myxomycetes and Basidiomycetes to groups peculiar to specific insect and microinvertebrate hosts.

The number of species of fungi associated with animals and animal products cannot be estimated because many species are host-specific and inconspicuous, and some fungal groups are poorly known world-wide. The known host specificity of arthropod-associated fungi and the vast diversity of arthropods in the Neotropics suggest that arthropod-associated fungi may be the largest in species diversity of all fungal trophic groups. A systematic search for all groups of animal-associated fungi in an area has never been undertaken.

### **11.2 Sampling for Fungi Associated with Animals and Animal Products**

Due to the broad array of host organisms, their life histories, and the diversity of habitats involved, a variety of methods is required to discover and identify fungi associated with animals and animal products. These methods are divided here into two groups. In the first set of methods (those that consist of direct observation), a fungal specimen is collected in the field or on animals or animal products brought back to the laboratory. Macrofungi on dung or animal remains may be collected by parataxonomists collecting terrestrial macrofungi (6.1, 6.2, 6.4, 6.5, and chapter 10). Minute Ascomycetes, Myxomycetes, etc. associated with animals or animal products will be located by laboratory para

axonomists. In some cases, isolation into culture is required for identification. However, many fungi associated with animals cannot be located by direct observation, either in the field or the laboratory. In the second group of methods (those that consist of indirect observation), fungi are first located by isolating them in culture from animals and animal products. This group of methods includes baiting of soil, water or other environmental samples with animals, animal parts or animal products, and then collecting or culturing of fungi that have colonized the baits. Specimens will also be incubated in moist chambers, and fungi developing on them will be collected or isolated into culture. Many of the organisms or their products to be used as baits or sources of animal-associated fungi will be provided by parataxonomists working for animal TWIGs. Coordination with activities of the various TWIGs working on animals will be important.

### **11.2.1 Specimens Collected in the Field**

Many animal-associated fungi can be located in the field by direct observation. Most conspicuous and well-known among these are insect-parasitic members of the Clavicipitales that fruit on larvae or pupae. These and other relatively conspicuous fungi including *Onygena* on animal horn, hair, hooves or feathers, and mushrooms fruiting on animal dung will be sampled by fungal parataxonomists collecting terrestrial macrofungi in all of the vegetation zones in the ACG. Branches of trees and shrubs in all habitats will be examined (8.4.2, 8.4.3) for members of the Septobasidiales on scale insects. Certain fungi, mainly members of the Ophiostomatales, are associated with the galleries of wood-boring beetles. These become established in the galleries and play a role in the nutrition of the beetle larvae. Fallen trees of most of the species to be sampled for fungi associated with wood (chapter 9) will be examined for beetle galleries and pieces of substrate collected when it is established that fungi are present. Some of the fungi present may be identified by direct observation, and others will require culturing or induction of fruiting bodies in moist chambers. These collections will be supplemented with specimens donated by researchers collecting various animal taxa.

### **11.2.2 Fungi Obtained in the Laboratory by Examination of Animal and Animal Product Specimens**

A variety of less conspicuous fungi are associated with animals and animal products of arthropods. Primarily they belong to four orders: (1) Ascosphaerales, (2) Entomophthorales, (3) Laboulbeniales, and (4) Septobasidiales.

The Ascosphaerales occur in the nests of bees where they cause a disease of the larvae called “chalk brood”. Nests of bees will be examined for these fungi. Although less conspicuous than the Clavicipitales, the Entomophthorales

can also be recognized in the field and collected by parataxonomists working with insects. Members of the Laboulbeniales are microscopic, and are probably the most numerous of all arthropod parasites. These will be found by the direct examination of specimens of insects and arachnids collected as part of inventories of those organisms. Larvae of aquatic insects are parasitized by *Coelomomyces* and other aquatic fungi. These will be assessed by directly examining material collected by members of the TWIGs studying those organisms.

Parasites of nematodes, other mesofauna, and microfauna are numerous throughout the world. Most of these will be found by cultural methods, but some may be located and identified by direct examination of individuals captured during Nematode TWIG studies and maintained in Petri dish cultures. Fungal specimens from nematodes will be processed as described in 6.3 and 7.4.

Fungi mutualistic with vertebrates are known, but mostly unstudied. The best known of these are anaerobic symbionts occurring in the stomachs of ruminants and the truffles and truffle-like fungi that are consumed by vertebrates. Spores of truffles and other gut-associated fungi will be sought by direct microscopic observation of the gut contents of vertebrates captured during the Vertebrate ATBI. Specimens will be processed as described in 6.3 and 7.4. Gut aerobes and anaerobes will be sampled by specialized techniques (11.3.2j).

### **11.2.3 Other Fungi Obtained in the Laboratory**

Fungi parasitic on vertebrates will be enumerated by direct examination of captured animals and by autopsy. Work on fungi associated with terrestrial vertebrates will be carried out by a medical mycologist while that on fish will be done by a fish pathologist. Cultures will be established as necessary for identification of the causal fungi (6.3, 7.3, 7.4).

## **11.3 Protocols for Obtaining Fungi Associated with Animals and Animal Products**

### **11.3.1 Field Collecting by Parataxonomists**

#### **Requirements**

- a Macrofungi and Myxomycetes associated with animals and animal products (particularly dung) shall be collected and processed according to 6.1, 6.2, 6.4, and 6.5.
- b Fallen trees, particularly those felled for the Fungus ATBI (chapter 9) shall be examined for beetle galleries and pieces of substrate from these galleries shall be collected when it is established that fungi are present.
- c Samples from beetle galleries (11.3.1b) shall be processed according to 6.3 and 6.4.

Note: Moist chambers will also be used with regard to these samples to induce sporulation (11.3.2h).

### 11.3.2 Isolation of Fungi from Animals and Animal Products

#### Requirements

Note: All cultures are to be preserved in the INBio culture collection (7.3.2).

- a Vertebrates, arthropods, mesofauna and microfauna collected during activities of animal TWIGs shall be used for isolating fungi occurring as external or internal parasites or carried on the animals' surfaces.
- b Nests, dung, bones, hair, and other animal products collected during activities of animal TWIGs shall be used for isolating fungi occurring in these products or on their surfaces.
- c Seven groups of methods shall be used for obtaining fungi associated with collected animals:
  - i washing, combing, scraping, and swabbing surfaces and orifices of animals and plating dilutions (11.3.2d)
  - ii autopsy (11.3.2e)
  - iii incubation of living mesofauna and microfauna (11.3.2f)
  - iv baiting (11.3.2g)
  - v incubation of animal parts (11.3.2h)
  - vi dilution plating or plating of washed particles from animal products (11.3.2i)
  - vii dilution plating of gut contents (11.3.2j).
- d Methods of washing, combing, scraping, and swabbing the surfaces and orifices of animals and plating dilutions on nutrient media for nonaquatic organisms and in water for fungi on fish and other aquatic organisms shall be as follows:
  - i For vertebrates, samples shall include representatives of every species captured during routine work of the vertebrate TWIGs from the full variety of habitats in the ACG.
  - ii Arthropods, mollusks, annelids and macroinvertebrates shall be sampled using a selection representing five members of each family or other recognized major group, approximately 5,000 taxa, collected from the full variety of habitats in the ACG.
  - iii In the case of terrestrial animals, dilutions of swabbings shall be plated on Dilute Malt Extract Agar (7.2b(vii)) and DRBC Agar (7.2b(viii)) and incubated at 25 deg. C for 21 days.

- iv Dilutions of swabbings shall be incubated in water in the case of aquatic animals and processed as aquatic fungi according to 10.2.
- v Fungi associated with mesofauna and microfauna shall not be sampled by this method (see 11.3.2f).
- e Autopsy of diseased vertebrates by veterinarians or medical mycologists associated with the Parasites of Vertebrates TWIG shall lead to isolation of any fungi encountered using the methods described for isolation of fungi from macroinvertebrates (11.3.2d(ii)).
- f With regard to fungi associated with living mesofauna and microfauna captured by other TWIGs, the following shall be done:
  - i A selection of the collected living animals shall be maintained in vivo to enable direct observation and isolation of fungi developing from them.
  - ii Small living animals shall be maintained under observation in moisture chambers with appropriate substrates for one to six months or until associated fungi appear, and any such fungi shall be treated in the same manner as specimens obtained from the field.
  - iii The presence of fungi parasitic on mesofauna shall also be detected by placing likely substrates (e.g., rotting leaves) directly on unparasitized bait cultures of these organisms (Barron, 1977).
  - iv Axenic cultures of animal parasites shall be established for later study and identification; and specimens that appear to be unusual should be treated in the same manner as cultures isolated from other substrates.

Note: For some groups of fungi such as the Entomophthorales, cultures can be maintained only with great difficulty.

- g With regard to the use of dead animals and animal parts as bait, the following shall be done:
  - i Dead animals or animal parts shall be placed in situ in terrestrial sampling plots or in soil or water samples obtained from them.
  - ii Insects shall be attracted to odor-baited agar plates placed in terrestrial sampling plots throughout the ACG.
  - iii Parasites of aquatic insects or their stages shall be sought by baiting pond and stream water from various habitats with ap-

proprate insects provided by arthropod TWIGs; and isolation from the baits shall use the methods of 10.2.

- iv Cadavers of a variety of vertebrates shall be buried at the edge of several sampling plots in each habitat type and monitored periodically for the presence of mushrooms.

Note: Although whole dead vertebrates are not generally examined for fungi, these materials may become colonized by certain Basidiomycetes, especially if they are buried.

- v Small pieces of fish and whole invertebrates shall be submerged in samples of pond and stream water from all habitats. Chytrids and Oomycetes arising from these baits shall be isolated into pure culture following the methods of 10.2.3.
- vi Samples of soil and other materials from all habitats shall be baited with hair and feathers, and the resulting fungi shall be isolated following the methods of 7.3.1i.
- vii Recently killed insects shall be buried in soil chambers; and, after 1-5 months submergence in the chamber, the insects shall be exhumed, washed and plated on weak media. Resulting fungi shall be isolated for identification as detailed in Papierek and Hajek (1997).

Note: The media and methodology used for such isolation depends on the taxonomic group to which a given fungus belongs. For a member of the Clavicipitales or their asexual states, just plating spores on Cornmeal agar will work. For the Entomophthorales and other obligate parasites, specialized media are needed.

Note: Dead insects and other arthropods are probably decomposed in soil and other habitats by specialized fungi.

- h Decomposing and nondecomposing animal parts such as hair, horn, feathers, etc. and products of animal origin including dung, plant galls, leaf cutter ant middens, bird, termite and wasp nests, galleries of wood-boring beetles, mole and rodent burrows, vertebrate bedding materials, and vertebrate food caches shall be incubated in moist chambers from which fungi and Myxomycetes that develop shall be identified by direct observation or isolation into culture (11.3.2i).

Note: Keratinous materials such as hair, feathers, hooves, horns, etc. are decomposed by a specialized group of fungi, mostly members of the Onygenaceae. Many of these materials (especially those already decomposing when collected) are predicted to yield large numbers of fungal species.

- i Washed particles from animal parts and products (listed in 11.3.2h) and sites of particular animal visitation or activity such as slime fluxes on vascular plants shall be plated or dilution plated in order to isolate associated fungi.
- j Dilution-plated gut contents
  - i shall be carried out on nutrient media
  - ii shall be incubated under both aerobic and anaerobic conditions
  - iii shall be based on samples, as in 11.3.2d, that shall include every vertebrate species provided and, in addition, approximately 5,000 taxa representing five members of every family or major group of arthropods and macroinvertebrates.



## **Staff Resources of the Fungus ATBI—Tasks, Training, and Capacity Building**

The challenge of inventorying the array of fungi present in the ACG is considerable. Most of the responsibility for carrying out the Fungus ATBI will be assumed by Costa Rican scientists, either with or without a formal academic background, particularly the community of parataxonomists and curators associated with the Fungus TWIG. International experts will be involved in collecting while on visits to the ACG, but their primary function will be in capacity building and quality assurance of the work carried out by curators and parataxonomists. This approach has the considerable benefit of developing the expertise to utilize fully the intended products and to continue and extend activities related to ACG Fungus ATBI as well as to reduce the funds required to engage international experts. However, some essential expertise can be acquired only by funding the salaries and expenses of systematic experts who will be engaged to contribute to specimen identification and will be closely associated with the training of in-country personnel.

### **12.1 Tasks of the Fungus ATBI**

In comparison with other organisms surveyed in the ATBI, fungi are difficult to inventory because of the unavoidable need to detect inconspicuous species through direct field observation. Most other taxonomic groups with small individuals will be collected primarily using indirect sampling techniques. Both direct and indirect approaches must be employed in order to obtain knowledge of all fungi in this project.

Considerable responsibility and technical knowledge will be expected of the Fungus ATBI staff. Many of their tasks require extensive expertise in microscopy and aseptic technique, in recognition and description of fungal structures, and in an overall appreciation of fungal species concepts and systematics. The training component of the Fungus ATBI will therefore be a crucial part of the project.

#### **12.1.1 Tasks of Costa Rican and International Experts in the Fungus ATBI**

##### **Requirements**

- a Costa Rican and international experts shall be involved primarily in
  - i assigning scientific names, where possible, to the species prepared by Fungus ATBI curators

- ii training Fungus ATBI staff
- iii building capacity and efficiency of the Fungus ATBI staff
- iv developing and applying methods of assuring the quality of the work done by the Fungus ATBI staff (3.3.1g, 3.3.1i, 3.3.3a(ix)).

## **12.1.2 Tasks of Parataxonomists and Curators**

### **Requirements**

- a For the Fungus TWIG, parataxonomists and curators shall carry out the following tasks:
  - i collection of specimens, substrate samples, and recording all associated data
  - ii searching substrate samples for sporocarps
  - iii slide preparation of fungal samples following designated protocols
  - iv incubation and observation of samples in moist chambers
  - v preparation of substrate samples for indirect analysis
  - vi media preparation and surface sterilization
  - vii isolation and preparation of axenic (pure) cultures
  - viii study and description of cultures
  - ix photography and preparing drawings of fungal structures
  - x assigning specimens to morphological groups
  - xi checking and refining morphological groups into species
  - xii preparation of descriptions and illustrations
  - xiii where appropriate literature and expertise are available, identification of specimens
  - xiv herbarium curation
  - xv preservation of cultures by lyophilization, cryogenics, or agar slants
  - xvi data entry, editing, and analysis
  - xvii distribution of specimens and cultures to the user community.
- b It shall be the responsibility of each member of the staff to carry out his/her assigned tasks efficiently and effectively and continually to seek means by which the tasks may be carried out even more efficiently and more effectively.

## **12.2 Main Categories of Staff of the Fungus ATBI**

### **12.2.1 Categories of Staff of the Fungus ATBI**

#### **Requirements**

- a Two main categories of staff shall be established for the Fungus ATBI:
  - i parataxonomists (12.4, 12.5)
  - ii curators (12.6).

## **12.3 General Training for Staff of the ACG Fungus ATBI**

Many of the skills necessary for the development of a good fungal parataxonomist or curator will be developed during a general fungal parataxonomist/curator training course.

### **12.3.1 Fungal Parataxonomist/Curator Training Course**

#### **Requirements**

- a Upon completion of a fungal parataxonomist/curator training course, a participant shall
  - i have an understanding of the goals, term of work, and organizational structure of the ACG Fungus ATBI
  - ii have an understanding of the importance and role of the staff of the ACG Fungus ATBI
  - iii have developed and refined observational skills
  - iv have an understanding of the possible products of the Fungus ATBI (chapter 14)
  - v have an operational understanding of the general protocols defined in this Plan
  - vi have an understanding of the ecological zones of the ACG and of the habitats in which field work will be done
  - vii have an appropriate understanding of the forms of life for which the Fungus ATBI is searching and a basic understanding of the biology of these forms
  - viii have an appropriate understanding of the families of host plants associated with subject organisms of the Fungus ATBI
  - ix have an appropriate understanding of the vertebrates and the other families of host animals associated with subject organisms of the Fungus ATBI
  - x have an understanding of the role of isolation and culturing in the Fungus ATBI

Note: Training in specialized laboratory skills is covered in 12.5.1 and 12.5.2.

- xi define personal goals for education, skill development, and career definition during an expected period of work with the Fungus ATBI and after
- xii have a clear understanding of the importance of the quality of the work for which the staff will be responsible.

## **12.4 Parataxonomists—Field Training**

The number of parataxonomists needed for work in the field will vary significantly during the ACG Fungus ATBI. When the five-year full-scale ATBI begins, over 100 field taxonomists could be operating simultaneously at the height of field activity—if stop rules have not been implemented. Without results of the pilot program, the effect of stop rules is not clear. However, an extensive training program for field parataxonomists will be necessary and the courses will need to be repeated as attrition occurs. In addition, the use of mycotourists and other volunteers to aid in the sampling process suggests that a condensed form of the course must be developed and presented periodically in English as well as in Spanish.

### **12.4.1 Parataxonomists' Field-Related Tasks and Skills**

#### **Requirements**

- a In the field, parataxonomists shall spend most of their time gathering specimens and preparing field descriptions of these collections according to the protocols of this Plan to be analyzed by laboratory staff in Santa Rosa and INBio.
- b In the field, parataxonomists shall also be responsible for carrying out baiting protocols and observations of fungal succession in situ.
- c Tree-climbing skills shall be required of a number of field parataxonomists so that the collecting above ground level can be achieved.
- d As with parataxonomists assigned to other groups of small organisms, a detailed appreciation of subtle differences between sporocarps in the field, and between samples on plant and animal substrates shall be required.

### **12.4.2 Parataxonomists' Field Training**

#### **Requirements**

- a A field training module for fungal parataxonomists shall be developed to supplement the fungal training course for curators and parataxonomists with the goals defined in the present section. The length of the training module is proposed to be 4-weeks.

- b During the 4-week fungal parataxonomist field training module, at least 80% of the prospective fungal parataxonomists shall be given specialized training in fungal collecting protocols so that participants obtain the collecting and field note taking skills specific to certain groups of fungi.

Note: Collection of high quality, well-annotated specimens is critical. Because of the number of field parataxonomists, the variability in quality expected, and turnover in staffing, some specialized training and work in specific fungal groups will contribute to successful collection of high quality specimens.

- c Upon completion of the field training module a parataxonomist shall
  - i have a thorough operational understanding of the protocols specific to collecting, taking field notes, preparation of specimens in the field, photography of specimens, and procedures for transport of specimens to the appropriate laboratory or other facility
  - ii have experienced at least eight field trips during which sampling units shall be established and marked, plots shall be defined, plots shall be searched, fungi shall be collected, and all field parataxonomists' tasks shall be performed at least once
  - iii have been trained for and experienced collecting on living plants and on recently cut or dead trees
  - iv have been trained to distinguish lesions on leaves, stems, and roots that may indicate the presence of a fungal pathogen
  - v have been trained for and experienced leaf, bark, root, litter, water, and soil collection for use in indirect observation of fungi
  - vi have been trained for and experienced collecting in diverse aquatic habitats
  - vii have been trained for and experienced lichen collecting
  - viii have been trained for and experienced collecting of macro-mycetes with fleshy and perennial fruiting bodies
  - ix have been trained for collecting of Myxomycetes and, at least, experienced observation of Myxomycetes developed in moist chambers from bark samples
  - x be able to identify the most common plant hosts to family, genus, or species as is appropriate on a habitat by habitat basis.

Note: This training can largely be given by in-country experts, with some input from international fungal ecologists and systematists.

**Recommendations**

- d Parataxonomists working in the field should clearly understand the importance of stop rules (3.3.2) and assist in their development.

**12.5 Parataxonomists—Laboratory Training**

By the time stop rules are implemented and the volume of preliminary collecting is reduced and refined, analysis of specimens in the laboratory will occupy significantly more time than field collection. For this reason and to simplify communication and logistics, some parataxonomists will need both field and laboratory training.

**12.5.1 Parataxonomists' Laboratory Tasks and Skills****Requirements**

- a Parataxonomists' laboratory skills shall include
  - i microscopy
  - ii preparation of microscope slides of tissues of macrofungi and microfungi
  - iii observation of fungi in moist chambers and scanning of substrates under the dissecting microscope for fruiting structures of microfungi
  - iv isolation of fungi into pure culture
  - v maintenance of sterile conditions
  - vi control of infestation and contamination.
- b Laboratory parataxonomists shall sort collections into provisional species, and make preliminary observations and measurements.

**12.5.2 Parataxonomists' Laboratory Training**

The skills required by laboratory parataxonomists are considerable, and would normally be gained during an extended period of training in a mycological laboratory. To rapidly develop competence, an intensive eight-week course in San Jose is required with activities divided equally into laboratory practice and observational skills. Some laboratory components could in part be taught in conjunction with parataxonomist laboratory training for the Virus, Bacteria, and Alga TWIGs. Skill in observation and interpretation could be taught in partnership with preliminary training of curators.

**Requirements**

- a An intensive eight-week course shall be provided for laboratory parataxonomists with training divided equally between laboratory practice and observational skills.

## 12.6 Curators

### 12.6.1 Curators' Tasks and Skills

Curators will have responsibilities essential to the success of the fungal component of the ATBI and, in the best of circumstances, would have extensive experience in internationally recognized research centers. Curators will carry out almost all the duties of an experienced fungal systematist, trained over a period of at least five to ten years. The only systematic duties that they will not be required to perform on a regular basis are those of taxonomic specialists, namely authoritative identification of specimens. Because, at first, a large proportion of the fungi will either be undescribed or unrecognizable, access is required to a comprehensive herbarium and library; and sufficient resources of this kind may not exist within Costa Rica. A further significant part of the curators' responsibilities will be the on-going transfer of knowledge to the parataxonomists. This will result in continuous communication between the ATBI staff, allowing feedback of knowledge, and testing of keys and classification schemes.

#### Requirements

- a Curators shall have the aptitude for making accurate, careful observations both in the field and in the laboratory.
- b Curators shall be able to maintain organized records and manage simultaneously a variety of tasks.
- c Curators shall be computer literate.
- d Curators shall rapidly develop the capability to take numerous accurate recordings of macroscopic and microscopic characteristics of fungi, analyze the characteristics and compare them with those described in the literature in order to make identifications of common fungi, identify specimens to genus, and possibly develop expertise in specialized groups of fungi.

### 12.6.2 Curatorial Training

Twelve fungal curators are required for this project, reflecting both the enormous diversity of the fungi and the difficulty of their inventory. Some curators may already have sufficient training for their basic duties and additional training will be provided through in-country training by visiting experts. The discipline of mycological systematics has progressed rapidly in the last twenty years suggesting that even some established workers may need formal refresher courses. The majority of curators, especially those with responsibilities for mi-

crofungi, will need an extended period of intensive training in an international research center or centers with expertise in the fungi concerned.

### **Requirements**

- a A four-week preliminary course in San Jose shall be developed for those without established skills and background knowledge in mycology, partly in conjunction with parataxonomists laboratory training.
- b Such a preliminary course (12.6.2a) shall provide an introduction and an opportunity to determine the aptitude of prospective curators for the job.
- c Following the preliminary course, prospective curators shall be evaluated for their aptitude for the tasks involved in curation; and only those having sufficient aptitude shall be permitted to continue in the training beyond that course.
- d For those prospective curators who are judged to have the aptitude and knowledge to proceed in training (12.6.2c), a further period of three months (minimum) of full-time, out-of-country training, i.e., with teachers assigned exclusively to the student group, shall be established and provided.

Note: Three months of such training is the minimum necessary to produce staff with adequate skills for fungal curation duties. A relatively short period of intensive training will be of more value than an extended non-exclusive assignment to an expert's laboratory where the curators largely develop their own skills.

Note: Such training differs from the usual relationship between experts and their research assistants or associates at least in that much more information will have to be given directly to the trainees and more time will have to be spent directing and assessing their work than is normally the case with (for example) graduate students in research laboratories. Organizations and individuals providing training for Fungus ATBI curators should understand the unique requirements and intensive nature of the commitment.

## **12.7 Roles of Visiting Experts**

The interest in the ATBI project in the international systematics community suggests that many experts will request involvement in the inventory process in Costa Rica. Many of these scientists may expect to carry out variously focused sampling programs. These may uncover additional species for the inventory that would not otherwise result from the sampling protocols outlined in this Plan. Although this indirect contribution to the Fungus ATBI should in principle be encouraged, it is essential to stress three requirements.

## 12.7.1 Visiting Experts Interacting with the Fungus ATBI

### Requirements

- a Individual sampling by visiting experts not formally part of the Fungus ATBI shall be compatible with the organized sampling strategy being pursued by the parataxonomists and curators of the Fungus ATBI:
  - i All data derived from sampling not formally part of the Fungus ATBI shall be freely available to the Fungus ATBI in a timely manner—when the data are available and, often, prior to formal publication.
  - ii Visiting experts not formally part of the Fungus ATBI shall not take actions that might disrupt the sampling protocols or practices of the Fungus ATBI.
- b International experts not formally part of the Fungus ATBI shall be asked to contribute by training Fungus ATBI staff by a combination of formal teaching and informal knowledge transfer (mentoring), as well as by annotation of specimens.

## 12.8 Training of Specialists

Some of the fungal sampling and analytical techniques require training of specialists on an individual basis. These techniques include culturing of endomycorrhizal (VAM) fungi and acquisition and processing of molecular sequence data if molecular work is to be included as part of the ATBI. The training needs for these tasks are mentioned in the appropriate sections of the proposal (8.5.9u, Appendix C).

### 12.8.1 Curatorial Training with Relation to Arbuscular Mycorrhizal Fungi

With regard to endomycorrhizal (VAM) fungi, experience through extensive training is needed to establish and maintain cultures, to isolate and identify morphospecies, and to set up voucher specimens.

#### Requirements

- a A staff member reporting to the Culture Collection Manager (3.3.1) shall be selected to coordinate the staff working on culturing and identification of arbuscular mycorrhizal fungi; and this person shall receive two to three months of on-site training at INVAM (8.5.9) with the following goals:
  - i The staff member shall become proficient at extraction of VAM spores from samples.

- ii The staff member shall become proficient at culture development and preparation of permanent reference slides as vouchers.
- iii The staff member shall become able to read and understand species descriptions and use descriptions to make identifications.
- iv The staff member shall become able to use permanent vouchers of a reference collection in making identifications.
- v The staff member shall become able to thoroughly document observations and support those observations with digitized color images of representative specimens.
- vi The staff member shall become able to establish a permanent reference collection at INBio comprising permanent slides and digitized color images.
- vii The staff member shall become able to develop revised species descriptions for deposition at INBio and for use by others in Costa Rica and Central America.

## Bibliographic and Computer Resources for the Fungus ATBI

### 13.1 Basic Literature

In order to enable as many identifications as possible to be made in-country—either in the field, in the laboratory, or at INBio—a basic set of literature is essential. Using these resources, preliminary identifications can be made by generalists; and, for well-defined, cosmopolitan fungal species, minimal input will be needed from specialists. However, integrated literature for identification of most fungi simply does not exist, especially for little known, inconspicuous taxa. Indeed, even among larger agarics, few genera have modern monographs or up-to-date keys in any part of the Western Hemisphere—even in the more intensively studied areas such as the northeastern U.S. and southeastern Canada. Additionally, many of the taxa to be encountered by staff of the Fungus ATBI are anticipated to be new to science.

A limited number of the most useful key reference books are still in print. Most of the funding for literature must be spent on photocopies of articles and books that are no longer available. Arrangements for doing this may be made with international mycological libraries. Apart from literature for the curators, a set of general field guides will be needed for the parataxonomists in the field and laboratory. On the other hand, their needs also may be met by the development of photographic data bases as identification aids. At a minimum, the essential reference resources listed in Appendix A.1 must be obtained. *All* the references cited in the Plan (chapter 17) and in Appendix A would be useful for the ATBI library.

For the groups of fungi that include plant pathogens such as the Oomycetes, Zygomycetes, Ascomycetes, Deuteromycetes, Uredinales, and Ustilaginales, the most important references are listed in *A Literature Guide for the Identification of Plant Pathogenic Fungi* (Rossman et al., 1987). Those listed under the general references and marked as essential in this guide should be obtained for the Fungus ATBI.

For culture collection maintenance, the following is recommended: *The Preservation and Maintenance of Living Fungi* (Smith and Onions, 1994).

Hardcopy or on-line access is needed to current and back issues of three major mycological journals: *Mycologia*, *Mycological Research*, and *Mycotax-*

on. In addition, it would be valuable to have a complete set of *Micología Neotropical Aplicada*.

## 13.2 Computer Resources

Two electronic identification aids are available, namely *Polykey* for the identification of Aphyllophorales (Adaskaveg et al., 1994) and Kendrick's (1994) *Keys to the Genera of Hyphomycetes*. In addition, Internet access to international reference resources such as the data base of specimens in the U.S. National Fungus Collections is required.

### 13.2.1 World Wide Web Sites

A good site for access to general mycological resources is now available from the University of Kansas:

<http://www.keil.ukans.edu/~fungi/>

The URL for the U.S. National Fungus Collections web site is

<http://nt.ars-grin.gov>

A website concentrating on inventorying and monitoring sampling techniques is the following:

<http://nt.ars-grin.gov/documents/ross-biod/RSGroup.htm>

The Mycological Society of America's bulletin board includes a feature called "Mycology On-line" that includes a directory of email addresses for many mycologists. The MSA Bulletin Board can be accessed through

<http://www.erin.utoronto.ca./soc/msa/>

A site dedicated to Costa Rican agarics is maintained by the New York Botanical Garden:

<http://www.nybg.org/bsci/res/hall/costric.html/>

Among other information at this site, there are a number of digital images of Costa Rican agarics.

The Association for Tropical Biology maintains a website at

<http://www.ecology.umsi.edu/atb/>

The Organization for Tropical Studies has two websites, one in Costa Rica, as follows:

<http://www.ots.ac.cr/>

<http://www.ots.duke.edu/>

Announcements of new sites and URL changes for existing sites often appear in the MSA newsletter, *Inoculum*, which can be accessed via the MSA site.

### **13.2.2 ATBI Web Site**

The ATBI itself will have a web site, both for administrative activities and for access to products and data.



## 14

# Products

The Fungus ATBI will result in economic, educational, and scientific products beneficial to Costa Rica and the international community in the categories: a) education (14.1), b) tourism (14.2), c) research (14.3), d) forestry and agriculture (14.4), e) medicine (14.5), f) industry (14.6), and g) environment (14.7). Products specific to particular groups of fungi (14.8) are listed following these categories.

Many of the benefits of the Fungus ATBI listed below are products that could generate both local income and economic support for conservation in Guanacaste locally and Costa Rica nationwide.

### 14.1 Benefits to Education

Benefits from the Fungus ATBI will affect education at all levels, ranging from primary and secondary schools to colleges and universities and the general public of Costa Rica and the world. Similarly, many of the products listed under research will be of benefit in undergraduate and graduate education in colleges and universities. Among the educational products will be: field guides for mushrooms and other conspicuous fungi such as polypores and lichens. These books can be used in primary and secondary education as well as sold to tourists; books and computer or video programs documenting the diversity and natural history of unique and interesting fungal groups; basic educational texts illustrating the intricate ecological associations of all life forms; local and regional education programs through field trips and lectures; internship programs for secondary and college level students; training of local experts in fungal taxonomy; education of community leaders and government officials in the potential benefits and risks of biocontrol; development of environmental sensitivity, such as the essential interactions of plants with an array of fungal symbionts; materials for museum displays and exhibits, such as a leaf cutter ant nest with its associated fungal garden or collection, drying, and use as food of mushrooms; classroom activities such as growing mushroom gardens; illustrated books of beautiful fungi; and educational programs and books documenting the discovery of the fungal biodiversity.

### 14.2 Benefits to Ecotourism

Benefits to tourism will accrue from such products as posters, postcards, and consumer goods decorated with mushrooms or other fungi for sale at ACG and other museum shops; training of local naturalists and guides; generation of

resources useful in natural history interpretation (e.g., information on ecology, life cycles, distributions, mutualism and other interactions with the biota); and specific tourism opportunities for amateur mycologists (e.g., knowledge of edible species and their distribution).

### 14.3 Benefits to Research

Benefits to research and the research community are products of new or increased knowledge in three areas: taxonomy—identification and classification of organisms; increased fungal genetic resources at both cellular and subcellular levels; and ecology, or the roles of individual fungi and their interactions with other organisms in their environment. Many of the fungi discovered during the Fungus ATBI will be new or poorly-known, so a requirement of the Fungus ATBI will be the production of identification guides, manuals, computer data bases and keys to all major fungal groups, including tropical soil fungi, wood-decay fungi, plant pathogenic and plant-associated fungi, fungi associated with animals ranging from vertebrates through insect and microinvertebrates, aquatic and aero-aquatic fungi, and lichens. Some of these groups, such as chytrids and aquatic Oomycetes are problematic. Understudied groups of organisms and, in particular, tropical taxa have received only superficial study. Identification guides and increased knowledge will be of value in research, education, forestry and agriculture, and industry.

The Fungus ATBI will provide the first realistic measure of fungal species richness in relation to the diversity of other floral and faunal groups; a procedural model for assessing fungal diversity in other geographic areas and for other potential inventory projects; new and refined methods for collection, culture, enumeration and identification of fungal taxa; and comprehensive specimen and culture collections of Costa Rican fungi. Subsequent bioprospecting and physiological investigations of the fungi encountered and cultured in the Fungus ATBI will yield estimates of their relative physiological and taxonomic diversity. Because many of the lowland forest species present in the ACG are also found in other regions of North and South America, the Fungus ATBI will provide reasonable estimates of fungal species diversity on a larger scale.

Products of the Fungus ATBI of value in ecological research will include an increased understanding of the ecosystem functions of fungi (e.g., their roles in nutrient cycling, as ecto- and endomycorrhizae essential for the growth of plants, and as a source of nutrients for canopy insects), and a more fundamental understanding of their evolution, especially the co-evolution of symbiotic associations such as fungus-virus, fungus-plant, and fungus-insect associations. Manipulation of fungi, whether for eradication of agricultural pests or development of beneficial symbioses or products, will require knowledge of the fungal

associations with other organisms that act as their hosts, predators, and vectors or activators of propagule distribution and germination.

## **14.4 Benefits to Forestry and Agriculture**

Benefits to the agriculture and forestry sectors include control of plant diseases through better knowledge of plant pathogenic fungi and the role animals play in their distribution; vectoring of plant pathogenic viruses by fungi (in conjunction with the Virus ATBI); development of potential biocontrol agents for pathogenic fungi, insects, and noxious weeds causing crop losses; cultivation of edible mushrooms, including novel edible fungi for human or animal consumption that can be grown using agricultural, forestry or industrial wastes; organisms and products for food processing, such as better fermentation organisms and vegetarian sources of food processing enzymes and alternative food products; products for improved livestock feeds through delignification of woody wastes; improved plant nutrition through fungal (e.g., mycorrhizal) associations; and improved seed germination through fungal associations such as in orchid culture.

## **14.5 Benefits to Medicine**

Since the discovery of penicillin more than 50 years ago, natural products of fungal origin have played an important role in human affairs; fungi are one of the most significant sources of antibiotics, immuno-suppressant drugs and other pharmaceuticals such as antitumor and cholesterol-lowering agents. Recent emphasis on the use of biodegradable pesticides and the continued search for antibiotics and other medicines has revived interest in fungal metabolites. The large-scale isolation of fungi from unusual habitats, as envisioned in this project, will lead to the discovery of many novel substances. Other benefits to medicine include: increased knowledge of human fungal diseases, potential animal models, and sources of environmental inoculum of disease fungi; better understanding of the role of gut fungi in digestive processes; methods for the rapid identification of microorganisms, including emerging disease agents; and forensic science use of potential “corpse finder” mushrooms.

## **14.6 Benefits to Industry**

Benefits to industry include a source of organisms useful for biological control, promotion of plant growth, and regeneration of disturbed areas; sets of organisms that can be used as environmental indicators; a number of natural products, including antibiotics, cosmetics, dyes for fabric and paper, products for food processing, products for paper and wood processing, enzymes for biotechnology, and novel chemicals; and bioassays for specific metabolites.

## 14.7 Benefits to the Environment

Environmental benefits will include potential bioremediation of polluted areas using novel fungi to degrade carcinogenic, complex hydrocarbons and toxic wastes; baseline data useful for recording future changes in vegetation, climate, and air composition; use of mycorrhizal fungi in revegetation of nutrient-poor tropical soils; and use of wood-rotting and other fungi as indicators of ecological continuity of forests in the ACG or elsewhere in the Neotropics.

## 14.8 Products of Specific Fungal Groups

### 14.8.1 Animal-associated fungi

*Education.* Fungi associated with animals are among the most appealing for educational exhibits, illustrations and displays. Some examples are a leaf cutter ant nest with its associated fungus garden; mushrooms growing from buried cadavers; fungi such as *Cordyceps* attacking insects, nematodes or other minute fauna; fungus-animal interactions in the web of forest life (e.g., tiny shrews eating and distributing spores of false truffles that, in turn, are important mycorrhizal symbionts of giant forest trees); and fungus-animal interactions among plant pathogens, such as floral mimicry or chemical attractants of animal vectors.

*Agriculture and forestry.* Fungi are the most important agents of plant disease and cause losses in agriculture in the billions of dollars. Insects are thought to be important vectors of plant disease. An inventory of the plant pathogenic fungi occurring on the surfaces and guts of a variety of invertebrates will lead to a better understanding of how these fungi are spread and how they can be controlled.

The use of microorganisms in the biological control of insect pests is becoming increasingly important as agriculture shifts away from pesticides. An inventory of insect fungi is an important first step in discovering potential new agents of specific (“surgical”) biological control. A close collaboration between entomologists and mycologists is essential in an investigation of potential biological control agents.

Food fermentations using fungi are important in many Asian countries, but have yet to be exploited significantly in the western world. Because of their great physiological differences, fungi offer a vast potential for industrial processes. Of particular interest are fungi associated with animal food caches. These caches are often colonized by fungi and may well be improved in food value as a result of their enzymatic activities.

Invertebrate and vertebrate animals, including humans, benefit from a variety of microorganisms in their digestive tracts. These microorganisms aid in

the digestion of materials resistant to the enzymes of the animal itself, thus bringing about a more efficient use of food. Particularly notable for their digestive symbioses are ruminant animals such as cattle and certain insects, particularly termites. A systematic survey of gut symbioses in wild animals could lead to the discovery of organisms and processes useful in agriculture and industry.

*Medicine.* Fungi are the causal agents of a number of important diseases of humans and animals. In several cases pathogenic fungi such as *Histoplasma* and *Coccidioides* are known to occur among populations of wild animals. Knowledge of the distribution of fungal diseases among wild animals may lead to a better understanding of the origins and prevention of outbreaks among humans. Decay of vertebrate animals is generally thought to be the realm of bacteria, insects and worms; however, several fungi are known from this substrate and may be present in great abundance. Corpses of animals buried in soil may pass undetected until a mass appearance of mushrooms in the soil above them announces their presence. Such mushrooms, sometimes called “corpse finders,” are studied extensively in Japan, but have been ignored elsewhere. A knowledge of which fungi occupy cadavers in a variety of Costa Rican habitats could be an important contribution to forensic science throughout tropical America.

#### **14.8.2 Fungi Associated with Living Plants, Soils, and Plant Debris**

*Agriculture and forestry.* Mycorrhizae are mutualistic associations between fungi and plant roots. Most plants have mycorrhizae and these are essential to the well-being of the plant. Truffles, a group of edible underground fungi, are produced by specific mycorrhizal associations and are known to be dispersed by mammals and birds. Other less conspicuous mycorrhizal fungi also depend upon animal dispersal but these relationships are poorly understood. Since most mycorrhiza/animal associations involve passage of fungal spores through animal guts, examination of gut contents will shed light on how these mutualisms occur. Increased understanding and use of endomycorrhizal fungi—those obligately associated with plants growing in nutrient-poor tropical soils—is essential for the revegetation of deforested tropical areas by appropriate native species. Knowledge of the diversity of mutualistic and pathogenic fungi will be useful for comparing fungi in agricultural ecosystems with those in their native habitat. Although many of these plant pathogenic fungi are presumed to be host-species specific, this assumption has not been explored. The Fungus ATBI will be an opportunity to do so. Biotrophic fungi may prove useful as biological control agents for insects, plant pathogenic fungi and weeds. Guides to plant-associated fungi resulting from this project would be especially useful to plant pathologists in tropical regions.

*Medicine and industry.* Plant-associated fungi, especially endophytes, are a proven source of bioactive natural products useful as lead compounds for pharmaceutical and agrochemical development.

*Education.* Guides to the identification of tropical soil fungi are lacking, and would be a great benefit from the Fungus ATBI. Illustrated monographs of the aquatic and aero-aquatic Hyphomycetes of the ACG in English and Spanish will result from this project. Because these fungal species are widely distributed, this account may include a majority of such fungi in tropical regions. Aquatic and aero-aquatic fungi are especially important in primary decomposition of litter in fresh water habitats. A series of floristic or monographic treatments of chytrids, including algal parasites, could be produced.

### **14.8.3 Fungi Associated with Wood and Wood-decay**

*Agriculture and forestry.* One use of wood-decay fungi or their enzymes is for delignification of straw or other lignified agricultural or forestry wastes to improve their digestibility and quality for use as livestock feed. New and unusual wood-associated fungi may produce better strains and species useful in biological processes for lignin and hemicellulose removal from wood products for pulp and paper industries, eliminating the need for chemical processing and associated toxic waste production. Saprophytic wood-associated fungi are used in the biological control of root and trunk decay pathogens. Novel, edible fungi for human consumption can be grown using agricultural, forestry, or industrial waste products as nutrient sources.

*Environment:* Wood-decay fungi can break down a number of petroleum products and inorganic hydrocarbons. They are used to decontaminate soils and to detoxify industrial waste products including the by-products of the paper, plastic, and paint industries. Wood-rotting and other fungi can serve as indicators of the ecological continuity of forests in the ACG.

# 15

## Resource Needs

An estimated budget for the seven year Fungus ATBI is presented below. *Expenses that have not been budgeted* include the infrastructure costs: buildings including a cold room, network server for herbarium/culture collection, data base software for network, staff costs such as pensions and employment insurance, transport in the field and between San José and Guanacaste, and most administrative costs (e.g., telephones, staff recruitment and welfare contingencies, and inflation).

The proposed preliminary molecular research study (Appendix C) has been kept separate as it is clearly an extra (though very desirable). Many of the equipment costs and culture collection expenses might be eventually shared with other TWIGs.

Budget entries are in U.S. dollars.

The bottom line is as follows: Excluding proposed preliminary molecular research and the cost of having overseas specialists identify fungi, the estimated total cost is \$11.3 million. The cost of proposed molecular work is estimated at \$290,000. The high estimate for cost of specimen identification by overseas specialists is estimated at \$20 million. The highest over all cost is \$31.6 million.

### Head Mycologist

Coordination of Fungus ATBI (Head Mycologist)

$\$35,000/\text{year} \times 7 : \dots\dots\dots \$245,000$

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Total

$\dots\dots\dots \$245,000$

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### Field staff resources

Field work manager (Santa Rosa)

$\$14,000/\text{year} \times 7 : \dots\dots\dots \$98,000$

Field paratonomists for pilot program

$\$7,000/\text{year} \times 22 \times 2 \text{ years} : \dots\dots \$308,000$

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Total

$\dots\dots\dots \$406,000$

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Herbarium staff resources (San José)

Herbarium manager/taxonomist

 $\$20,000/\text{year} \times 7 \dots\dots\dots \$140,000$ Curator/taxonomists (3 Basidiomycete,  
7 Ascomycete - including mitosporic forms,  
1 Zygomycete - including endomycorrhizae,  
1 chytrid/protoctistan "fungi") $12 \times \$17,000/\text{year} \times 7 \dots\dots\dots \$1,428,000$ 

Administrative Officer

(loans, visitor liaison, etc.)

 $\$10,000/\text{year} \times 7 \dots\dots\dots \$70,000$ 

Laboratory parataxonomists

 $3 \times \$7,000/\text{year} \times 7 \dots\dots\dots \$147,000$ 

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Total $\dots\dots\dots \$1,785,000$ 

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Culture collection staff resources (San José)

Collection manager

 $\$20,000/\text{year} \times 7 \dots\dots\dots \$140,000$ 

Laboratory coordinators

 $2 \times \$17,000/\text{year} \times 7 \dots\dots\dots \$238,000$ 

Lab parataxonomists

 $15 \times \$7,000/\text{year} \times 7 \dots\dots\dots \$735,000$ 

Liquid nitrogen collection manager

(shared by all ATBIs)

 $0.2 \times \$14,000/\text{year} \times 7 \dots\dots\dots \$19,600$ 

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Total $\dots\dots\dots \$1,132,600$ 

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Herbarium equipment resources (San José)Herbarium cases (assumes an average of 5 specimens per species,  
1000 specimens per case) $(250 @ \$600) \dots\dots\dots \$150,000$ 

Specimen boxes for Basidiomycetes and other large fungi

 $(20,000 @ \$1) \dots\dots\dots \$20,000$ 

Freezing microtomes

 $(2 @ \$8,000) \dots\dots\dots \$16,000$

|  |             |
|--|-------------|
| PCs (basic specification)  |             |
| ( 15@\$1,500 )   | \$22,500    |
| PCs (high specification)   |             |
| ( 2@\$2,250 )  | \$4,500     |
| Laser printers   |             |
| ( 3@\$1,000 )  | \$3,000     |
| Image analysis system  |             |
| ( 2@\$15,000 )   | \$30,000    |
| Dissecting microscopes   |             |
| ( 20@\$3,000 )   | \$60,000    |
| Compound microscopes, including basic<br>photographic facilities |             |
| ( 12@\$9,000 )   | \$180,000   |
| Photomicroscope (Zeiss Axiophot)                                 |             |
| ( 2@\$80,000 )   | \$160,000   |
| Flatbed scanner  |             |
|  | \$1,000     |
| Herbarium sheets, glue, etc.                                     |             |
|  | \$50,000    |
| Slides, coverslips, stains, etc.                                 |             |
|  | \$320,000   |
| Slide boxes  |             |
|  | \$20,000    |
| TLC equipment  |             |
|  | \$6,000     |
| Miscellaneous consumables  |             |
|  | \$50,000    |
| <hr/>  |             |
| Total  |             |
|  | \$1,093,000 |
| <hr/>  |             |

#### Culture collection equipment resources (San José)

|                             |         |
|-----------------------------|---------|
| Peristolic pumps            |         |
| ( 3@\$2,000 )               | \$6,000 |
| Lab carts (stainless steel) |         |
| ( 10@\$150 )                | \$1,500 |
| Analytical balance          |         |
|                             | \$3,000 |

|  |           |
|--|-----------|
| Top loader balances                                    |           |
| ( 2@\$1,200 )  | \$2,400   |
| Laminar flow hoods                                     |           |
| ( 4@\$4,000 )  | \$16,000  |
| Autoclaves   |           |
| ( 2@\$25,000 )   | \$50,000  |
| Basic compound microscopes                             |           |
| ( 5@\$7,500 )  | \$37,500  |
| Dissecting microscopes                                 |           |
| ( 12@\$3,000 )   | \$36,000  |
| Micromanipulator stations                              |           |
| ( 2@\$7,000 )  | \$14,000  |
| Skerrman micromanipulators                             |           |
| ( 5@\$900 )  | \$4,500   |
| Incubators   |           |
| ( 5@\$20,000 )   | \$100,000 |
| Shakers  |           |
| ( 5@\$3,000 )  | \$15,000  |
| Refrigerators  |           |
| ( 5@\$500 )  | \$2,500   |
| Lyophilizers   |           |
| ( 2@\$14,000 )   | \$28,000  |
| Manifolds, torches, and pumps for above                |           |
|  | \$12,000  |
| Table top centrifuge                                   |           |
| ( 2@\$3,500 )  | \$7,000   |
| Automated Petri plate pourer                           |           |
|  | \$10,000  |
| Automated tube pourer                                  |           |
|  | \$2,000   |
| Small items (water baths, hot plates, glassware, etc.) |           |
|  | \$100,000 |
| PCs (basic grade)                                      |           |
| ( 10@\$1,500 )   | \$15,000  |
| PC (high specification)                                |           |
|  | \$2,250   |
| Laser printers   |           |
| ( 3@\$1,000 )  | \$3000    |
| Miscellaneous consumables                              |           |
|  | \$100,000 |

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Total

..... \$567,650

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Field staff resources (Guanacaste) for full-scale Fungus ATBI
Terrestrial fungi

Field parataxonomists

 $28 \times \$7,000/\text{year} \times 5 \dots\dots\dots \$980,000$ 

Laboratory parataxonomists

 $5 \times \$7,000/\text{year} \times 5 \dots\dots\dots \$175,000$ 
Wood-associated fungi

Field parataxonomists

 $18 \times \$7,000/\text{year} \times 5 \dots\dots\dots \$630,000$ 

Lab parataxonomists

 $2 \times \$7,000/\text{year} \times 5 \dots\dots\dots \$70,000$ 
Aquatic fungi

Field/lab parataxonomist

 $1 \times \$7,000/\text{year} \times 3 \dots\dots\dots \$21,000$ 
Living plant-associated fungi

Field parataxonomists

 $8 \times \$7,000/\text{year} \times 5 \dots\dots\dots \$280,000$ 

Lab parataxonomists

 $10 \times \$7,000/\text{year} \times 5 \dots\dots\dots \$350,000$ 
Endophytic fungi

Lab parataxonomists

 $10 \times \$7,000/\text{year} \times 5 \dots\dots\dots \$350,000$ 
Animal-associated fungi

Field parataxonomists

 $2 \times \$7,000/\text{year} \times 5 \dots\dots\dots \$70,000$ 

Lab parataxonomists

 $9 \times \$7,000/\text{year} \times 5 \dots\dots\dots \$315,000$ 


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Total

..... \$3,241,000

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Field equipment resources (Guanacaste)
General equipment

Media

 $\$50,000/\text{year for 5 years} \dots\dots\dots \$250,000$

|  |           |
|--|-----------|
| Plastic/glassware                          |           |
| \$20000/year for 5 years .....             | \$100,000 |
| Other lab consumables                      |           |
| \$5000/year for 5 years .....              | \$25,000  |
| Refrigerators                              |           |
| ( 6@\$500 ) .....                          | \$3,000   |
| Compound microscopes (shared)              |           |
| ( 5@\$15,000 ) .....                       | \$75,000  |
| Microscope consumables                     |           |
| .....                                      | \$15,000  |
| Laminar flow cabinets                      |           |
| ( 10@\$6,000 ) .....                       | \$60,000  |
| Specimen freezer                           |           |
| .....                                      | \$500     |
| Miscellaneous packets, boxes, labels, etc. |           |
| .....                                      | \$50,000  |
| Autoclave                                  |           |
| .....                                      | \$5,000   |
| Specimen storage cases                     |           |
| ( 5@\$600 ) .....                          | \$3,000   |

Note: Media prepared in San José, only minimal facilities needed in Guana-  
caste

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|   |           |
|---|-----------|
| Subtotal                                  |           |
| .....                                     | \$586,500 |
| <u>Terrestrial fungi</u>                  |           |
| Dissecting microscopes                    |           |
| ( 5@#3,000 ) .....                        | \$15,000  |
| PCs (basic specification)                 |           |
| ( 5@\$1,500 ) .....                       | \$7,500   |
| Specimen driers                           |           |
| ( 10@\$150 ) .....                        | \$1,500   |
| GPS units                                 |           |
| ( 10@\$1,000 ) .....                      | \$10,000  |
| Cameras with macro lenses and flash units |           |
| ( 10@\$750 ) .....                        | \$7,500   |
| Film                                      |           |
| .....                                     | \$55,000  |

Radios

( 10@\$300 ) ..... \$3,000

Rucksacks

( 10@\$100 ) ..... \$1,000

Hand lenses

( 20@\$20 ) ..... \$400

Supplies for field collecting and

storing specimens (plastic boxes with

movable dividers (ca 30 × 15 × 3cm) for

protection of delicate specimens, aluminum

foil, waterproof paper, knives, specimen boxes,

litter rakes, plastic bags, and herbarium packets

..... \$20,000

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Subtotal

..... \$120,900

Wood-associated fungi

Cameras with macro lenses and flash units

( 5@\$750 ) ..... \$3,750

GPS units

( 5@\$1,000 ) ..... \$5,000

PCs (basic specification)

( 2@\$1,500 ) ..... \$3,000

Dissecting microscopes

( 2@\$3,000 ) ..... \$6,000

Specimen driers

( 3@\$300 ) ..... \$900

Film

..... \$10,000

Rucksack

( 5@\$100 ) ..... \$500

Hand lenses

( 10@\$20 ) ..... \$200

Knives, saws, etc.

..... \$900

Radios

( 5@\$300 ) ..... \$1,500

Miscellaneous bags, etc.

..... \$10,000

---

Subtotal

..... \$41,750

Aquatic fungi

Vacuum pump

..... \$2,000

Refrigerator

..... \$500

Dissecting microscope

..... \$3,000

Tools, nets, bags, vials, etc.

..... \$1,500

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Subtotal

..... \$7,000

Living plant-associated fungi

Collecting bags, plastic bags, etc.

..... \$20,500

Refrigerated specimen packs

( 20@\$20 ) ..... \$400

Rucksacks

( 6@\$100 ) ..... \$600

Hand lenses

( 20@\$20 ) ..... \$400

Knives, saws, etc.

..... \$900

Specimen driers

( 3@\$300 ) ..... \$900

Cameras with macro lenses and flash units

( 5@\$750 ) ..... \$3,750

Film

..... \$25,000

GPS units

( 6@\$1,000 ) ..... \$6,000

Radios

( 6@\$300 ) ..... \$1,800

|                        |                |
|------------------------|----------------|
| Dissecting microscopes |                |
| ( 10@\$3,000 )         | ..... \$30,000 |
| PCs (basic)            |                |
| ( 10@\$1,500 )         | ..... \$15,000 |
| Drying presses         |                |
| ( 20@\$10 )            | ..... \$200    |
| Moist growth chambers  |                |
| ( 200@\$4 )            | ..... \$800    |

---

Subtotal

..... \$106,250

## Endophyte studies

|                               |                 |
|-------------------------------|-----------------|
| Agar (300 kg@\$2,000/kg)      |                 |
|                               | ..... \$600,000 |
| Petri dishes                  |                 |
| ( 90,000@\$.50 )              | ..... \$45,000  |
| Slants                        |                 |
| ( 1,800,000@\$.24 )           | ..... \$432,000 |
| Refrigerators                 |                 |
| ( 10@\$500 )                  | ..... \$5,000   |
| Laminar flow cabinets         |                 |
| ( 10\$6,000 )                 | ..... \$60,000  |
| Miscellaneous lab consumables |                 |
|                               | ..... \$5,000   |

Note: Collections made by living plant field staff with Plant ATBI bearing cost.

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Subtotal

..... \$1,147,000

## Animal-associated fungi

|                         |                |
|-------------------------|----------------|
| Microscopes: dissecting |                |
| ( 8@\$3,000 )           | ..... \$24,000 |
| Petri dishes            |                |
|                         | ..... \$10,000 |
| Bottles                 |                |
|                         | ..... \$15,000 |
| Media                   |                |
|                         | ..... \$12,000 |

|                           |                   |
|---------------------------|-------------------|
| Specimen driers           |                   |
| ( 2@ \$300 )              | ..... \$600       |
| Film                      |                   |
|                           | ..... \$5,000     |
| Miscellaneous consumables |                   |
|                           | ..... \$40,000    |
| <hr/>                     |                   |
| Subtotal                  |                   |
|                           | ..... \$106,600   |
| <hr/>                     |                   |
| Total                     |                   |
|                           | ..... \$2,115,400 |
| <hr/>                     |                   |

Visiting scientist costs

|  |                 |
|--|-----------------|
| Terrestrial fungi  |                 |
| 5 × \$3,000 for 7 years:   | ..... \$105,000 |
| Aquatic fungi  |                 |
| 4 × \$3,000 for 7 years:   | ..... \$84,000  |
| Wood-associated fungi  |                 |
| 2 × \$3,000 for 7 years:   | ..... \$42,000  |
| Living plant-associated fungi  |                 |
| 4 × \$3,000 for 7 years:   | ..... \$84,000  |
| Animal-associated fungi  |                 |
| 2 × \$3,000 for 7 years  | ..... \$42,000  |
| Foreign expertise for identification<br>of difficult taxa, etc. (4 years full-time equivalent) |                 |
|  | ..... \$320,000 |
| <hr/>  |                 |
| Total  |                 |
|  | ..... \$677,000 |
| <hr/>  |                 |

GRAND TOTAL EXCLUDING MOLECULAR STUDY AND IDENTIFICATION BY EXPERTS

..... \$11,263,250

MOLECULAR STUDY (one-year preliminary research study only)

Developing methods

|   |                |
|---|----------------|
| Postdoc in established international lab (1 yr) |                |
|   | ..... \$17,000 |
| PC  |                |
|   | ..... \$1,500  |

|                                       |           |
|---------------------------------------|-----------|
| Consumables                           |           |
| .....                                 | \$15,000  |
| Training costs                        |           |
| .....                                 | \$10,000  |
| Travelling and subsistence expenses   |           |
| .....                                 | \$20,000  |
| <hr/>                                 |           |
| Subtotal                              |           |
| .....                                 | \$63,500  |
| <u>Applying methods in Costa Rica</u> |           |
| Salary                                |           |
| .....                                 | \$17,000  |
| Microfuge                             |           |
| .....                                 | \$2,000   |
| Speedvac and lyophilizer              |           |
| .....                                 | \$5,000   |
| Electrophoresis equipment             |           |
| .....                                 | \$2,000   |
| Automated sequencer                   |           |
| .....                                 | \$100,000 |
| Freezer, refrigerator                 |           |
| .....                                 | \$2,500   |
| Super speed centrifuge                |           |
| .....                                 | \$15,000  |
| Clinical centrifuge                   |           |
| .....                                 | \$1,500   |
| Shaking incubator                     |           |
| .....                                 | \$4,000   |
| Standard incubator                    |           |
| .....                                 | \$500     |
| PCR machine                           |           |
| .....                                 | \$12,000  |
| Milli-Q system                        |           |
| .....                                 | \$10,000  |
| Vortexers                             |           |
| .....                                 | \$500     |
| Pipetteman                            |           |
| .....                                 | \$3,000   |
| Transilluminator/image analysis       |           |
| .....                                 | \$7,500   |

|                               |           |
|-------------------------------|-----------|
| Hybridization chamber         | \$3,000   |
| Water baths                   | \$1,000   |
| Miscellaneous glassware, etc. | \$10,000  |
| Enzymes and biochemicals      | \$30,000  |
| <hr/>                         |           |
| Subtotal                      | \$226,500 |
| <hr/>                         |           |
| Total                         | \$290,000 |

GRAND TOTAL INCLUDING MOLECULAR STUDY,  
BUT EXCLUDING IDENTIFICATION BY EXPERTS  
..... \$11,553,250

IDENTIFICATION OF SPECIMENS BY EXPERTS  
(ASSUMING ALL COLLECTIONS IDENTIFIED BY  
OUT OF COUNTRY EXPERTS)  
(50,000 species each represented by 20 specimens @\$20 per specimen)  
..... \$20,000,000

GRAND TOTAL FOR FUNGUS TWIG  
..... \$31,552,650

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# Appendix A

## References on Isolation and Identification of Fungi

### A.1 Essential References

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# Appendix B

## Latin American and Spanish Participation in the Fungus ATBI

The Latin American countries and Spain have highly qualified mycologists with expertise in specialized groups, who could collaborate in the Fungus ATBI project by training parataxonomists, curators, and/or identifying fungal specimens. Although the complete list of such mycologists is not available, a partial list of these scientists is presented here by country.

### *Argentina*

|                   |                                     |       |
|-------------------|-------------------------------------|-------|
| Daniel Cabral     | Ascomycetes, endophytes, soil fungi | (UBA) |
| Mario Rajchenberg | Aphyllophorales                     | (UTF) |
| Andrea Romero     | Ascomycetes, endophytes             | (UBA) |
| Verónica Suárez   | Basidiomycetes                      | (UBA) |
| Jorge Wright      | Basidiomycetes                      | (UBA) |

### *Brazil*

|                 |                                |       |
|-----------------|--------------------------------|-------|
| Robert Barreto  | Biocontrol fungi, Hyphomycetes | (UFV) |
| José Dianese    | Plant-inhabiting fungi         | (UB)  |
| Ludwig Pfenning | Soil fungi                     | (UB)  |
| Katia Rodríguez | Ascomycetes, endophytes        | (IOC) |

### *Cuba*

|                       |  |       |
|-----------------------|--|-------|
| Hugo Iglesias Brito   | Lichens (Cladoniaceae)   | (IES) |
| Mayra Camino Vilaro   | Myxomycetes (Stemonitales, Trichiales)                           | (JBN) |
| Sara Herrera Figueroa | Aphyllophorales (Polyporaceae,<br>Ganodermataceae, edible fungi) | (IES) |
| Eduardo Furrázola     | Endomycorrhizae  | (IES) |
| Angel Mercado-Sierra  | Hyphomycetes   | (IES) |
| Julio Mena Portales   | Hyphomycetes   | (IES) |

|                          |                                       |       |
|--------------------------|---------------------------------------|-------|
| Jorge Nelis Blanco       | Tricholomataceae, Agaricaceae         | (IES) |
| Luis Ortiz Medina        | Tricholomataceae, Agaricaceae         | (IES) |
| Gloria Recio Herrera     | Ascomycetes (Xylariales)              | (JBN) |
| M. Rodríguez Hernandez   | Ascomycetes (Meliolales, Capnodiales) | (JBN) |
| Kendra Rodriguez Morejon | Biology, cultivation of microfungi    | (IES) |

### ***México***

|                          |                                      |               |
|--------------------------|--------------------------------------|---------------|
| Víctor Bandala Muñoz     | Herbarium techniques, Basidiomycetes | (IE)          |
| Gloria Carrión           | Ascomycetes                          | (IE)          |
| Santiago Chacón          | Ascomycetes                          | (IE)          |
| Ignacio Chapela          | Ant fungi                            | (ERA)         |
| Joaquín Cifuentes Blanco | Agaricales                           | (UNAM)        |
| Arturo Estrada Torres    | Macromycetes                         | (Tlaxcala)    |
| Gastón Guzmán            | Basidiomycetes                       | (IE)          |
| Laura Guzmán Davalos     | Agaricales                           | (UG)          |
| Gabriela Heredia         | Hyphomycetes (Leaf litter fungi)     | (IE)          |
| Alejandro Kong Luz       | Basidiomycetes                       | (Tlaxcala)    |
| Leticia Montoya Bello    | Basidiomycetes                       | (IE)          |
| Dulce Salmenes           | Edible Mushrooms                     | (IE)          |
| Lucía Varela Fregoso     | Macromycetes                         | (Politecnico) |
| Luis Villarreal          | Macromycetes                         | (IRGPT)       |

### ***Spain***

|                            |                             |       |
|----------------------------|-----------------------------|-------|
| María Núñez                | Aphylllophorales            | (UO)  |
| José María Egea            | Lichens                     | (UM)  |
| José Guarro                | Ascomycetes, Hyphomycetes   | (URV) |
| Francisco de Diego Calonge | Gasteromycetes, Ascomycetes | (JBM) |
| Carlos Lado                | Myxomycetes                 | (JBM) |
| María Teresa Tellería      | Aphylllophorales            | (JBM) |
| Gabriel Moreno             | Agaricales                  | (UAH) |
| Ricardo Galán              | Discomycetes                | (UAH) |
| Enrique Descals            | Aquatic Hyphomycetes        | (UPM) |

|                         |                 |       |
|-------------------------|-----------------|-------|
| José Canon              | Dung Fungi      | (URV) |
| María José Figueras     | Oomycetes & TEM | (URV) |
| <b><i>Venezuela</i></b> |                 |       |
| Teresita Iturriaga      | Ascomycetes     | (USB) |

***Key to Abbreviations***

|      |  |
|------|--|
| ERA  | Estudios Rurales y Asesoría  |
| IE   | Instituto de Ecología, Jalapa, México                                |
| IES  | Instituto de Ecología y Sistemática de Cuba                          |
| IOC  | Instituto Osvaldo Cruz, Sao Paulo                                    |
| IRGP | Instituto de Recursos Genéticos y Producción Tropical                |
| JBM  | Jardín Botánico de Madrid  |
| JBN  | Jardín Botánico Nacional, Cuba                                       |
| UAH  | Universidad de Alcalá de Henares, Depto. de Biología Vegetal, Madrid |
| UB   | Universidade de Brasilia   |
| UBA  | Universidad de Buenos Aires  |
| UFV  | Universidade Federal de Viçosa                                       |
| UG   | Universidad de Guadalajara   |
| UM   | Universidad de Murcia  |
| UNAM | Universidad Nacional Autónoma de México                              |
| UO   | University of Oslo, Norway   |
| UPM  | Universidad de Palma de Mallorca, Depto. de Biología Ambiental       |
| URV  | Univ. Rovira i Virgili, Reus, Tarragona                              |
| USB  | Universidad Simon Bolívar  |
| UTF  | Universidad de la Tierra del Fuego                                   |



## Appendix C

# Sampling Protocols—A Molecular Approach

Because most fungal species can be observed only through some kind of technological manipulation, their described number has been steadily increasing as new technologies for baiting, culturing and visualizing them have been deployed. Because there is no mycological equivalent of the light-trap for insects, the fungal species described to date have been discovered using a variety of techniques that are primitive by any measure. Up to now, there has been no objective means by which to measure the proportion of existing fungal diversity that can be recovered by the traditional means of direct observation and culturing. Examples of fungal groups in environments that would not be fully explored with the sampling techniques described elsewhere in this Plan include anaerobic, ruminant gut fungi; specialized kleptoparasites of ant nests; unculturable fungi from mycangia, mycetocytes, and insect galls; thermophiles in nests of herpetofauna; algal parasites in aquatic habitats; and anaerobic yeasts in fermenting fruits. Within the scope of the Fungus ATBI, but not detected by protocols presented here, there may be a significant number of fungal species.

One approach to obtaining some crude estimate of this cryptic realm of fungi would be to obtain an estimate of the diversity of fungal-specific DNA in the region of interest. Although a molecular approach cannot replace the assessment of fungal diversity based on the methods described elsewhere in this report, it could run in parallel to this assessment with three goals:

- a) to establish point estimates of the proportion and diversity of “unculturable/unobservable” fungi in selected habitats in the Fungus ATBI
- b) to help discern taxonomic units (“species”) in particularly recalcitrant groups (e.g. endomycorrhizae, Labyrinthulales; *Phomopsis*, Plasmodiophorales)
- c) to provide an independent measure of the proportion of fungal species detected by all other means.

### C.1 A Molecular Measure of Fungal Biodiversity

Given the ecological and taxonomic breadth of fungi to be encountered in the Fungus ATBI, we recommend the establishment of both preliminary and comprehensive projects on molecular approaches to the project. Simple sequence-oriented molecular methods provide the only available approach toward an all-encompassing inventory of fungal diversity.

### C.1.1 Molecular Measure of Fungal Biodiversity—Methodologies

#### Requirements

- a Simple Sequence Sieve. The Simple Sequence Sieve method
  - i shall make use of sets of primers with nested specificity—such as short tandem repeats
  - ii shall result in selection of a subset of amplified molecules that can be assessed for heterogeneity in order to obtain an estimate of the number of distinct taxa in the sample.

Note: Ideally this method would reflect the sum of the total diversity of fungal taxa in the sample.

Note: The simple sequence sieve procedure may be affected by factors other than the primer specificity or sequence of the annealing sites. It may be affected by the length of the region to be amplified, the presence of inhibitor substances in the DNA sample, and the reaction conditions.

Note: DNA may become contaminated unless care is taken to maintain a molecular laboratory with restricted use.

- b Clonal Trap. The Clonal Trap method
  - i shall make use of amplified sequences using a preliminary fungus-specific primer set
  - ii shall result in products that shall be cloned, sequenced, and examined for sequence diversity and fungal-specific sequence markers.

Note: This approach is limited by the number of clones obtained. In addition, sequences that escape cloning will not be detected.

- c If recommendations C.1.1d and C.1.1e are implemented then the following preliminary actions shall be required:
  - i A research program shall determine which DNA regions are best to target for amplification and sequence analysis.
  - ii A research program shall determine which fungal-specific markers can be used to identify these DNA regions (C.1.1c(i)) as fungal sequences.
  - iii A research program shall carry out the design of primers for amplification.

Note: The above programs will require extensive expertise, and a preliminary research project is needed to test the efficacy of the methods.

#### Recommendations

- d Total DNA from samples of selected habitats should be extracted and a defined region of DNA (e.g. rDNA) should be amplified.

Note: In an ideal fungus inventory, further sequencing-based steps would be devised to further separate taxa until a desired level of resolution is achieved to determine species-equivalent taxa. This process would be carried out iteratively, with continuous reference to cultured and observed fungi from the same habitat in order to achieve a degree of resolution at an assumed level of genetic diversity that might be considered equivalent to a taxonomic unit or species.

- e Two molecular approaches a Simple Sequence Sieve method (C.1.1a) and a Clonal Trap method (C.1.1b) should be used in combination.

Note: These two approaches are not mutually exclusive. Each one will provide useful information to advance the other. However, emphasis on either approach at the expense of the other can strongly influence the products obtained.

- f If only one molecular method is used, it should be the Simple Sequence Sieve method (C.1.1a).

Table 4. Comparison of Simple Sequence Sieve and Clonal Trap Methods

|   | Simple Sequence Sieve  | Clonal Trap                                       |
|---|--|---|
| Maximum number of terminal taxa                           | Major portion of genomes in sample                             | All clones obtained                               |
| Selection of taxonomic units (TUs) counted                | TUs not predetermined  | TUs determined by choice of cloned material       |
| Training and technical development                        | Mostly in international set-up (development of primers)        | More in-country training and development possible |
| Gross economics (assuming technology/methodology in hand) | Shorter (analysis can be stopped and still provide some info.) | Longer (analysis must be carried to end)          |
| Facility requirements                                     | No microbiological capacity                                    | Microbiological capacity                          |
| Immediate applicability                                   | Only applicable if efficient primer set available (long term)  | Fewer pre-project design steps                    |

## C.1.2 Discussion

A comparison of the likely output and the associated resources required for each of the two methods presented above (C.1.1a, C.1.1b) is presented in Table 4.

### **C.1.3 Potential Benefits and Products of a Molecular Measure of Fungal Biodiversity**

New sequence-based technology will be developed, such as novel methods for assessing sequence diversity of largely unknown organisms. This technology will be applicable to the biotechnology community as a whole and to other TWIGs with recalcitrant taxa.

Basic information on the phylogeny of poorly understood organisms will be made more accessible, addressing fundamental questions in evolution.

It may be possible to test current methodology for sequenced-based phylogeny estimation.

Methods may be developed for rapid identification of microorganisms, including potential plant and animal pathogens.

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